Using Biochemical and Structural Approaches to Study EGFR-family Heterodimers

by

Lily Raines

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Abstract

The epidermal growth factor receptor (EGFR/ErbB1) is a receptor tyrosine kinase that is activated upon the formation of specific ligand-bound dimers. There are four homologs of EGFR in humans, and dysregulation of any of the ErbB's is associated with various types of cancer. Of particular interest are ErbB3, which has an essentially inactive kinase domain, and ErbB2, which does not bind ligand. ErbB2 is overexpressed in approximately 20% of breast cancers, and heterodimers that contain ErbB2 bind their partner ligands more tightly, are recycled more slowly, and are more active than other ErbB hetero- and homodimers. ErbB2 is the preferred binding partner for the other ErbB's as well, and ErbB2/3 heterodimers can evade ErbB2 targeted therapies. Given the clinical relevance of the EGFR family and of ErbB2 in particular, the following studies were pursued.

First, nearly full length EGFR (tEGFR) isolated *in vitro* was packaged into nanodiscs for enzymatic characterization. Previous work with tEGFR revealed interesting facets of wild-type and mutant tEGFR activation, but concerns that detergent solubilized tEGFR would not be as active as if it were in the cell cast doubt on the relevance of this work to physiological EGFR activity. Nanodiscs were used to isolate tEGFR in a membrane-like bilayer, where it was shown to be as active as when detergent solubilized. Nanodiscs were also used to package Smoothened, type I insulin-like growth factor receptor (IGF1R) and ghrelin-O-acyltransferase (GOAT) for other work, demonstrating our mastery of this packaging technique that is broadly useful for studying membrane proteins.

Second, ErbB2-containing heterodimers are of clear physiological relevance but no structural work has been done to determine how they evoke such powerful cellular responses. The ErbB2 extracellular domain (ECD) is structurally different from the other ErbB's and thus a likely source for this different behavior. To study these ECD heterodimers, a tethering scheme was developed where the heavy (IgG) and light (IgK) chains of an Fab were each fused to one ErbB ECD by flexible glycine-serine linkers. Principally this work deals with ErbB2-IgK and ErbB3-IgG fusions. This scheme produces soluble, stable ErbB family heterodimers that bind ligand and thus are suitable candidates for subsequent structural studies.

Finally, Fab-tethered ErbB heterodimers were subjected to crystallization trials, small angle X-ray scattering (SAXS), and negative stain electron microscopy (EM) so that we might better understand the structure of an ErbB2-containing ECD heterodimer and how ErbB3 conformational dynamics are affected within an ErbB2/3 dimer. Unfortunately, exhaustive crystallization efforts did not yield crystals of sufficient quality for X-ray structural determination of either ErbB2/3 or ErbB2/EGFR heterodimers in the presence and absence of relevant ligands and therapeutic antibodies. Similarly SAXS and EM were unable to address our questions about ErbB3 ECD conformational dynamics. However, this Fab-tethering scheme will be broadly applicable to other heterodimers of interest and cryo-EM may yet yield the structural information we sought.

Advisor: Daniel J. Leahy

Reader: Philip A. Cole

Acknowledgements

I could not have completed this thesis without the love and support of my family and friends, particularly the support of my mother Muffy, my father Victor, and my brother Jack Raines. I thank Michael Donovan for helping me through my most difficult days and for helping me celebrate my successes. This work is dedicated to you all.

Table of Contents

List of Figures

List of Tables

Chapter 1. Nanodiscs and Enzymology

Acknowledgements

Zhihong Wang, Thomas Cleveland, Kwangsoo Kim, and Philip Cole were all critical mentors for this project.

Introduction

Nanodiscs, developed by the Sligar group (Ritchie et al., 2009), were used to isolate purified, nearly-full length truncated epidermal growth factor receptor (tEGFR) for enzymatic studies. The enzymatic properties of detergent solubilized wild-type and cancer associated tEGFR mutants had been previously studied by the Leahy and Cole groups(Qiu et al., 2009)(Wang et al., 2011), but there were concerns that this system may not reproduce the activity of EGFR *in vivo* owing to solubilization of tEGFR in detergent micelles. I modified the general protocol for packaging single transmembrane proteins into MSP1E3D1 nanodiscs from Ritchie et al. and the protocols described by the Springer group for EGFR packaging(Mi et al., 2008) and subsequently characterized the enzymatic activity of tEGFR in nanodiscs. The results of this work were published in 2013 as part of larger work describing Mig6 regulation(Wang et al., 2013), and the manuscript of this paper is reproduced below.

Type I insulin-like growth factor receptor (IGF1R) and ghrelin-O-acyltransferase (GOAT) were also packaged into nanodiscs for negative stain electron microscopy and enzymatic studies, respectively. Although IGF1R images were not ultimately used for structural studies and GOAT packaged in nanodiscs was inactive (as it was when detergent solubilized), the methods for packaging these proteins are included below.

This work was published in ACS Chemical Biology in 2013. The full information for this paper is:

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Abstract

Under physiological conditions, epidermal growth factor receptor (EGFR) tyrosine kinase activity is tightly controlled through the coordinated action of both positive and negative regulators. Aberrant EGFR activation occurs frequently in many cancer types, and the endogenous EGFR feedback inhibitor, Mig6/RALT, is more efficiently phosphorylated by oncogenic EGFR variants. We have utilized expressed protein ligation to generate semisynthetic Tyr394 phosphorylated and unphosphorylated forms of the Mig6 protein and shown that phosphorylation of Mig6 reduces its ability to inhibit purified, near full-length EGFR (tEGFR). We also demonstrate that the kinetic parameters of tEGFR are similar whether solubilized in detergent or reconstitutued in nanodisc bilayers. These findings suggest a mechanism by which EGFR and its family members evade negative regulation by Mig6 under pathological conditions.

Results and Discussion

The epidermal growth factor receptor (EGFR) signaling cascade has been one of the most intensively studied signaling pathways in human biology and is the target of several clinically approved anticancer drugs(Lemmon & Schlessinger, 2010)(Hynes & Lane, 2005). EGFR and its family members HER2/Neu, HER3, and HER4 are composed of several domains including an extracellular growth factor- binding domain, a

transmembrane domain, an intracellular tyrosine kinase domain, and a C-terminal tail that can be autophosphorylated. Binding of EGF to EGFR stimulates its tyrosine kinase activity via an allosteric mechanism involving formation of an asymmetric dimer of intracellular kinase domains(Zhang, Gureasko, Shen, Cole, & Kuriyan, 2006). This asymmetric dimer juxtaposes the C-lobe of a donor kinase domain with the N-lobe of an acceptor (activated) kinase domain. Activating mutations of EGFR are frequently observed in cancer, including the common alterations L858R and exon 19 (aa746−750) deletion(Paez et al., 2004)(Pao et al., 2004). The protein Mig6 (RALT) was identified as an endogenous inhibitor of EGFR signaling(Ferby et al., 2006)(Anastasi, Baietti, Frosi, Alemà, & Segatto, 2007) and loss of Mig6 function can augment tumor development in animal models(Ferby et al., 2006). Biochemical analysis of Mig6 has revealed that a 77 amino acid (aa) region (aa 336−412) of Mig6 is responsible for EGFR inhibition. This 77 aa region can be further subdivided into two segments, segment 1 (aa 336−364) and segment 2 (aa 365−412). An X- ray crystal structure of Mig6 segment 1 complexed with the EGFR kinase domain shows an extended surface of interaction of segment 1 with the kinase C-lobe that spans a region critical for forming the asymmetric dimer with the N-lobe of the other subunit(Zhang et al., 2007). Mig6 segment 1 thus physically obstructs asymmetric dimer formation, thereby inhibiting EGF-mediated EGFR activation.

Although the structure of Mig6 segment 2 is uncertain, the Mig6 segment 1 + 2 polypeptide is ∼100-fold more potent than Mig6 segment 1 alone in inhibiting EGFR kinase activity(Zhang et al., 2007)(Wang et al., 2011). It is speculated that Mig6 segment 2 interacts with the EGFR kinase domain along a path approaching the active site, thereby augmenting the affinity of the C-lobe targeted segment 1 for EGFR.8 The

oncogenic L858R and exon 19 (aa746−750) deletion EGFR variants appear to be resistant to Mig6 inhibition, presumably because their asymmetric dimer is more strongly favored(Wang et al., 2011).

Whether and how Mig6 itself is regulated has been unresolved. Mass spectrometry studies showed that Mig6 is tyrosine phosphorylated in cells, and phosphorylation of Tyr394 is enhanced in lung cancer cell lines carrying oncogenic L858R or exon 19 (aa 746−750) deletion mutations as well as in cells that overexpress HER2/Neu(Guha et al., 2008)(Wolf-Yadlin et al., 2006). Biochemical studies with purified proteins showed that EGFR can directly tyrosine phosphorylate Mig6(Wang et al., 2011). As Tyr394 is located within segment 2 of Mig6, it is conceivable that Mig6 phosphorylation could modulate its inhibition of EGFR, which is the subject of this study.

Using in vitro kinase assays with purified, solubilized tail- truncated EGFR (aa 25−1022, tEGFR), His6-Mig6 segment 1 + 2 appears to be phosphorylated on multiple Tyr residues since Y394F/Y395F Mig6 was still efficiently phosphorylated by tEGFR (Supplementary Figure 1). Producing a singly phosphorylated preparation of pTyr394-Mig6 using tEGFR was thus not possible. We turned instead to protein semisynthesis and the method of expressed protein ligation(Muir, Sondhi, & Cole, 1998)(Szewczuk, Tarrant, & Cole, 2009)(Vila-Perelló & Muir, 2010)(Hackeng, Griffin, & Dawson, 1999). In this approach, a recombinant protein fragment containing a C- terminal thioester is generated by the action of a stalled intein, and this thioester is chemoselectively ligated to an N-Cys containing peptide to generate a standard amide bond at the ligation junction. After several attempts, we were unable to get adequate soluble His6-Mig6-intein fusion

protein production. We next investigated a GST-Mig6 (aa 336−391) intein fusion, and this gave acceptable production of GST-Mig6 segment 1 + 2 after ligation with N-Cys synthetic peptides aa 392−412 with and without phosphorylation at Tyr394 (Figure 1.1a, Supplementary Figure 2). We were able to obtain ~80% conversion to ligated products by carrying out the reaction at 4 °C. The minor unligated GST-Mig6 may have arisen from nonspecific proteolysis or thioester hydrolysis that would prevent protein ligation. Semisynthetic proteins after purification by ion exchange and size-exclusion chromatographies showed a minor impurity of unligated GST-Mig6, equally represented in both phosphorylated and unphosphorylated preparations (Figure 1.1b). Since we showed that unligated GST-Mig6 (IC50 > 3 μM) is only a weak inhibitor of EGFR relative to ligated GST-Mig6 segment 1 + 2 (Supplementary Figure 3a), we presume the minor contaminant of unligated GST-Mig6 to be inconsequential. Semisynthetic GST-Mig6 in phosphorylated and unphosphorylated forms showed the correct molecular weights using MALDI-TOF mass spectrometry (see Methods). The phosphorylation status of semisynthetic phospho-GST- Mig6 was further confirmed by Western blotting with anti-pTyr antibody (4G10) as shown in Figure 1.1c.

To analyze the effects of semisynthetic GST-Mig6 on EGFR activity, we used solubilized, purified tEGFR, which we have described previously(Qiu et al., 2009), as well as tEGFR reconstituted in membrane bilayer nanodiscs(Bayburt & Sligar, 2009). Nanodiscs were used to test whether detergent solubilization of tEGFR alters its enzymatic properties relative to a native membrane environment (Figure 1.2a). The steady-state kinetic parameters including apparent k_{cat} and K_m values with a synthetic peptide substrate of tEGFR were similar when comparing the nanodisc and detergent-

solubilized forms (Figure 1.2b and Supplementary Figure 4). Likewise, inhibition by unphosphorylated semisynthetic GST-Mig6 segment 1 + 2 was similar for the nanodisc and solubilized tEGFRs with an IC_{50} of 0.3−0.4 μM (average of two independent assays) (Figure 1.3 and Supplementary Figure 3b). Moreover, since we replaced native aa 390−2 (Ser-Ser- Thr) with Pro-Gly-Cys to facilitate the expressed ligation reaction for GST-Mig6 semisynthesis (see Methods), it was important to assess the effects of these substitutions on the potency of Mig6 inhibition of tEGFR. Fortunately, these aa 390−2 replacements were shown to be well-tolerated as the unphosphorylated, semisynthetic GST-Mig6 showed nearly identical inhibitory potency (IC_{50} of 0.3−0.4 μM) toward tEGFR as the standard recombinant His6-Mig6 segment 1 + 2 that was wild-type (WT) in aa 390−2 (Supplementary Figure 5). Remarkably, Tyr394-phosphorylation of GST-Mig6 conferred a sharp reduction in inhibitory potency, with $IC_{50} \approx 3$ μM for pTyr394-GST-Mig6 against both solubilized and nanodisc-reconstituted tEGFR (Figure 1.3 and Supplementary Figure 3b). This weakened potency was comparable to that of unligated GST-Mig6 (aa 336−391) (Supplementary Figure 3a), underscoring that pTyr394 phosphorylation is quite disruptive of the contributions of Mig6 segment 2 to EGFR inhibition. Since the segment 1 region (aa 336−364) in both unphosphorylated and phosphorylated GST-Mig6 is identical, and this region alone has previously been shown to bind to the C-lobe of EGFR kinase domain with weak inhibition against EGFR ($IC_{50} \approx$ 100 μM)(Zhang et al., 2007),(Wang et al., 2011), it is speculated that the interaction between EGFR kinase domain and Mig6 segment is maintained after phosphorylation of Tyr394 (Figure 1.4). Interestingly, the His6-Mig6 mutant Y394E (IC_{50} of 0.74 μM) was only 2-fold less potent than unphosphorylated WT His6-Mig6 (IC_{50} of 0.35 μM),

indicating that the Glu side chain is a poor mimic of a phosphotyrosine in this case (Supplementary Figure 5). Thus, it would have been difficult to discern the authentic function of Tyr394-phosphorylation of Mig6 using mutagenesis and standard cell-based transfection experiments.

These results suggest that tyrosine kinase-catalyzed phosphorylation of Mig6 likely represents a feedback mechanism to antagonize its inhibition of EGFR (Figure 1.4). As noted, at least two EGFR family members when hyperactivated by mutation (EGFR) or elevated by increased expression (HER2/Neu) appear to target Tyr394 for phosphorylation in cells(Guha et al., 2008)(Wolf-Yadlin et al., 2006). Thus, Mig6 phosphorylation provides a mechanism for these kinases to override the inhibition mediated by Mig6. Such an effect would augment the signaling and cell proliferative properties associated with the EGFR family kinases, all of which are believed to be susceptible to Mig6 inhibition. Thus, the activation of one family member can bolster the signaling from each of the others. Whether other phosphorylation sites on Mig6 also modulate its function will be an interesting direction for future study.

While the structural details of the interactions of Mig6 segment 2 with EGFR are not well understood, it is reasonable to speculate that segment 2 extends near the kinase domain active site where Tyr394 can be targeted for modification. Furthermore, we hypothesize that Tyr394 phosphorylation of Mig6 causes electrostatic repulsion or sterically clashes with the substrate ATP and/or the catalytic base aspartate. As has been seen with other kinases, phosphorylation of this substrate peptide motif can reduce affinity for the kinase, enhancing product release(Kim & Cole, 1998),(Cook, Neville, Vrana, Hartl, & Roskoski, 1982). Thus, it will be interesting to investigate whether

phosphorylation of endogenous protein inhibitors of kinases represents a general mechanism for overriding their inhibition. Such on/off switches may help account for the highly rapid cellular phosphoprotein dynamics often observed in signaling cascades(Qiao, Molina, Pandey, Zhang, & Cole, 2006)(Kleiman, Maiwald, Conzelmann, Lauffenburger, & Sorger, 2011).

These studies also provide a cautionary lesson for using Glu residues as mimics of phosphotyrosine(Xia et al., 2008),(Tamada, Farrell, & Zallen, 2012). While the negative charge of the Glu can nominally substitute for the anionic pTyr, its smaller size, reduced charge, and different shape potentially limit its utility in this regard.

The use of nanodisc reconstituted tEGFR shows that the key features of the kinase activity seen in the detergent solubilized tEGFR are recapitulated in the lipid bilayer-embedded protein. Thus, the transmembrane helices, which have recently been suggested to be crucial for transmitting signaling information from the ectodomain to the intracellular region(Red Brewer et al., 2009),(Scheck, Lowder, Appelbaum, & Schepartz, 2012),(Endres et al., 2013), (Adak, Yang, Macdonald-Obermann, & Pike, 2011), appear to be functional in the detergent solubilized systems. It will be interesting in future studies to determine if different lipid compositions in nanodiscs affect EGFR kinase activity.

Materials and Methods

From Wang et al.

Plasmid Construction.

DNA encoding the N-terminal truncated Mig6 segment 1 + 2 (56 amino acids, residues 336−391) with an N- terminal GST-fusion and two mutations, S390P and

S391G, was subcloned into the pTYB2 vector (New England Biolads) to yield pTYB2-GST-Mig6 plasmid. Initial semisynthetic Mig6 experiments showed that the two native C-terminal Ser-Ser residues of Mig6 gave poor yields of thioester product and impeded subsequent ligation. As also noted in the NEB brochure for IMPACT vectors, C-terminal Pro- Gly has been reported to be very efficient for thioester generation and expressed protein ligation. Plasmid pT7HT-His6-Mig6 encoding Mig6 segment 1 + 2 (77 aa, residues 336−412) with an N-terminal His6 tag was prepared previously and contained WT 390−2 residues.9 Using Quikchange mutagenesis, the mutations Y394E, Y394F, and Y395F were introduced into His6-Mig6 segment 1 + 2, respectively.

Peptide Synthesis.

The C-terminal fragment of Mig6 (21 aa, residues 392−412) and EGFR peptide substrate (Biotin-RAHEEI- YHFFFAKKK-COOH) were synthesized using the standard Fmoc solid phase peptide synthesis strategy described previously(Tarrant et al., 2012). Two Mig6 peptides were synthesized, one without any post-translational modification, termed the unphosphorylated Mig6 peptide (CHYY- LLPERPPYLDKYEFFR), and the other containing a phosphorylated tyrosine residue to replace the native Tyr394, termed the phosphorylated Mig6 peptide (CHpYYLLPERPPYLDKYEKFFR). After C-18 reversed phase HPLC purification, synthetic peptides were >95% pure, and structures were confirmed by mass spectrometry.

Mig6 Semisynthesis.

Plasmid pTYB2-GST-Mig6 was trans- formed into E. coli BL21 (DE3) codon plus cells. Transformed cellswere cultured at 37 °C until A600 reached 0.6, and then the culture was incubated at 16 °C for 20 h in the presence of 500 μMIPTG to induce

expression of GST-Mig6 (aa 336−391)-intein-CBD fusion protein. Cell pellets were resuspended in lysis buffer (50 mM HEPES pH 7.4, 400 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 tablet of protease inhibitor cocktail (Roche)) and lysed by passage through a French Pressure cell three times. After centrifugation at 27,000g for 30 min to remove cell debris, the supernatant was collected and incubated with chitin beads (New England Biolabs) for affinity purification. The chitin beads bound with GST-Mig6-intein-CBD fusion protein was washed thoroughly with 1 M NaCl in washing buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 0.1% (v/v) Triton X-100), followed by one quick wash with 1 M urea in washing buffer. After equilibration with three times the bead volume of cleavage buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mMEDTA), the chitin beads were incubated with 200 mM 2- mercaptoethane sulfonate (MESNA, Sigma-Aldrich) in cleavage buffer at 25 °C for 16 h to release the GST-Mig6 protein with a C-terminal thioester. Then, 0.5 mM phosphorylated Mig6 peptide (CHpYLLPERPPYLDKYEKFFR) was added to the cleaved GST-Mig6 thioester solution, and the ligation reaction was allowed to proceed for 48 h at 4 °C. Upon ligation reaching 80% completion as estimated by Commassie-stained SDS-PAGE, the semisynthetic phosphorylated GST-Mig6 seg 1 + 2 was further purified with Mono Q ion exchange chromatography and Superdex 75 size-exclusion chromatography. The unphosphorylated and phosphorylated forms of semisynthetic GST- Mig6 seg 1 + 2 were obtained using the same conditions. The incorporation of the phosphorylated tyrosine residue was verified with Western blot using the antiphosphotyrosine antibody 4G10 (Millipore). The purity and concentration of GST-Mig6 seg 1 + 2 were determined with SDS-PAGE Coomassie staining and Bradford assay using bovine serum albumin as a standard. The unligated GST- Mig6 was obtained

by omitting the peptide ligation step. To confirm semisynthetic GST-Mig6 with mass spectrometry, GST-Mig6 was further purified by reversed-phase HPLC on a C-4 column. The peak corresponding to pure GST-Mig6 was collected and analyzed by MALDI-TOF. The masses of the semisynthetic proteins were internally calibrated versus the unligated GST-Mig6 protein (calcd. m/z: 32,505). The unphosphorylated semisynthetic GST-Mig6 showed m/z 35,287 (calcd. m/z: 35,283), and the phosphorylated semisynthetic GST-Mig6 showed m/z 35,334 (calcd. m/z 35,363). The estimated standard error on these m/z measurements is ±50 Da.

Packaging of EGF/tEGFR into Nanodiscs and EGFR Kinase Assays.

Our protocol for packaging EGF/tEGFR into nanodiscs is modified from the methods of the Sligar and Springer groups(Ritchie et al., 2009)(Mi et al., 2008). L-α-Phosphatidylcholine isolated from chicken eggs (Avanti Polar Lipids) that had been suspended in chloroform was dried under nitrogen to produce a film on the interior of a disposable borosilicate glass culture tube. Residual chloroform was removed by keeping this film under vacuum overnight. This lipid film was resuspended in a sodium cholate solution (200 mM sodium cholate, 20 mM Tris-HCl, 0.1 M NaCl, 0.5 mM EDTA, pH 7.4) to a molar ratio of cholate to lipid of 2:1. Detergent solubilized EGF/tEGFR, expressed and purified as described above, and membrane scaffold protein (MSP1E3D1) were added to this solution to a final molar ratio of EGF/tEGFR to MSP1E3D1 to lipid to cholate of 1:10:1590:3180. These molar ratios resulted in packaging of EGF/tEGFR in ~10% of nanodiscs formed. This solution was diluted with buffer (20 mM Tris-HCl, 0.1 M NaCl, 0.5 mM EDTA, pH 7.4) to the desired final volume for the packaging reaction and was incubated for 60 min at 4 °C. Then, 250 mg of Bio- Beads (BioRad), polystyrene

adsorbent beads that remove detergent from solution, were added per mL of reaction volume and incubated overnight at 4 °C. Biobeads were removed by centrifugation, as described previously(Ritchie et al., 2009), and the final reaction mixture was filtered and stored at 4 °C until use.

Additional Nanodisc Packaging Protocols

Smoothened

The packaging reaction was carried out as described as above, where 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids) was used instead of Egg PC to a final ratio of lipid:MSP of 69.5:1. The final molar ratio of Smoothened to MSP1E3D1 to lipid to cholate used for this reaction was 1:10:695:1390. Smoothened was provided by Thomas Cleveland. The success of this reaction is shown in Figure 1.5

IGF1R

The packaging reaction was carried out as described as above, where 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids) was used instead of Egg PC to a final ratio of lipid:MSP of 75:1. MSP1D1, which should yield discs with diameter of 9nm, was also used instead of MSP1E3D1. The final molar ratio of IGF1R to MSP1D1 to lipid to cholate used for this reaction was 1:10:750:1500. IGF1R was provided by Jennifer Kavran. The success of this reaction is shown in Figure 1.6

GOAT

MSP1E3D1 was used with DMPC at a lipid:MSP of 129:1. The final molar ratio of GOAT to MSP1E3D1 to lipid to cholate used for this reaction was 1:10:1290:2580. GOAT was provided by Martin Taylor. The success of this reaction is shown in Figure 1.7.

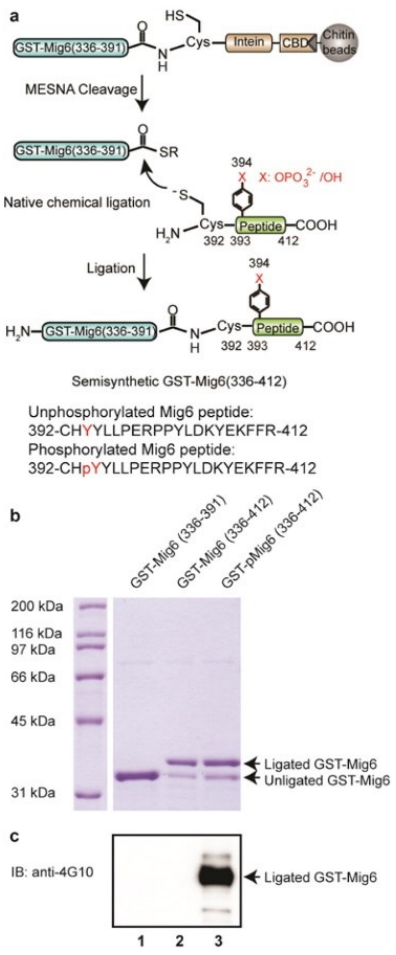
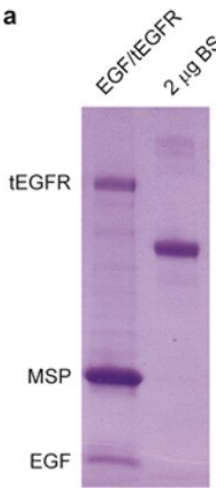


Figure 1.1 Preparation of the GST-Mig6 semisynthetic proteins by expressed protein ligation (EPL). (A) Scheme for EPL to generate the semisynthetic GST-Mig6 segment 1 + 2 containing phosphorylated tyrosine in a full stoichiometry. The residue Tyr394 that can be phosphorylated by tyrosine kinases is highlighted in red. (B) Coomassie stained 10% SDS-PAGE of unligated and semisynthetic GST-Mig6 proteins. (C) The incorporation of phosphorylated Tyr394 into semisynthetic GST-Mig6 was verified with Western blot using the antiphosphotyrosine antibody 4G10. The same amount of unligated (1), unphosphorylated (2), and phosphorylated (3) Mig6 proteins obtained via expressed protein ligation were analyzed by 10% SDS- PAGE and probed with 4G10 antibody. Figure from Wang et al, 2013.

a



b

EGF/tEGFR	K_m^{app} Peptide (μM)	K_m^{app} ATP (μM)	k_{cat} (min^{-1})
in detergent	12 ± 2 [a]	8.9 ± 1.7	11.7 ± 0.4
in nanodiscs	14.8 ± 2.9	6.0 ± 0.9	10.2 ± 0.2

Figure 1.2 Reconstitution of purified EGF/tEGFR into phospholipid bilayer nanodiscs. (A) WT tEGFR (aa 25−1022) purified from transiently transfected HEK293 GnTi− cells in the presence of 0.03% dodecyl maltoside was reconstituted into nanodiscs assembled with membrane scaffold protein (MSP) and egg phosphatidylcholine lipids. The reconstituted EGF/tEGFR was subjected to 4−20% gradient SDS-PAGE and Coomassie staining to verify the incorporation of EGF/tEGFR into nanodiscs. BSA is used as a standard to quantify the protein concentrations. (B) The kinetic parameters of reconstituted EGF/tEGFR and EGF/tEGFR in detergent were compared. aPre- viously reported data. Enzymatic parameters (±SD) were calculated from duplicate experiments. Figure from Wang et al, 2013.

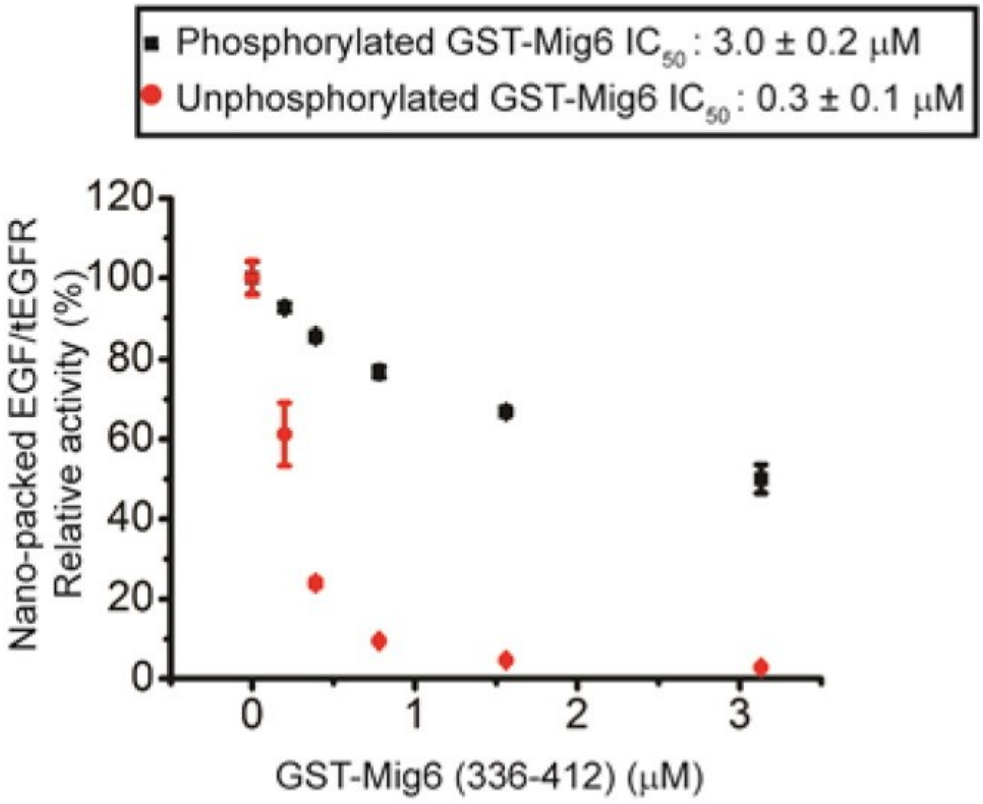


Figure 1.3 Distinct inhibitory effects of semisynthetic unphosphorylated and phosphorylated GST-Mig6 on EGF/tEGFR reconstituted in nanodiscs. IC_{50} values of Mig6 were determined by quantifying the relative kinase activity of EGF/tEGFR in the presence of varying concentrations of unmodified and phosphorylated GST-Mig6 (0, 0.20, 0.39, 0.78, 1.56, and 3.13 μM). Figure from Wang et al, 2013.

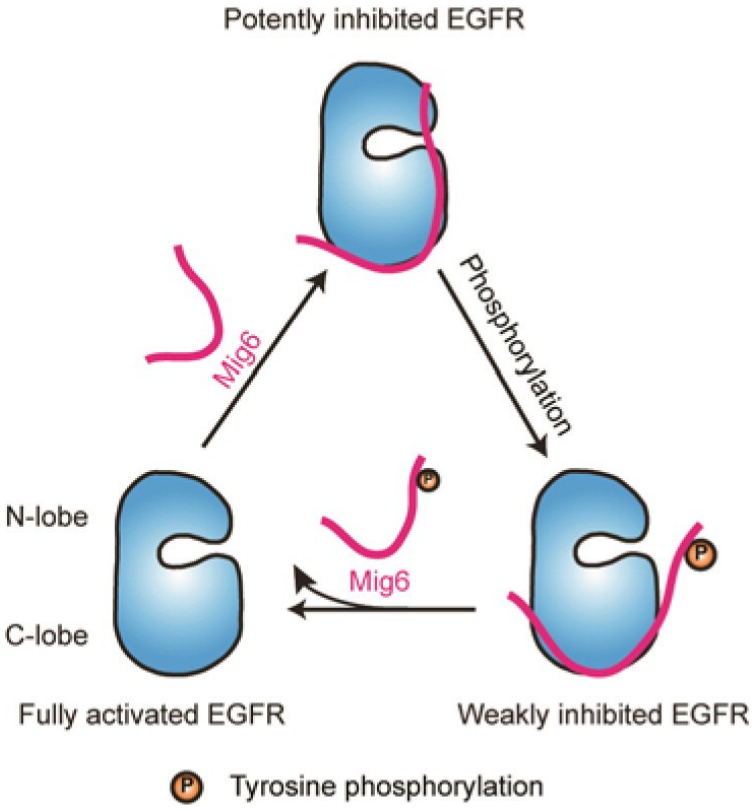


Figure 1.4 Feedback regulation of EGFR activation by Mig6, an endogenous inhibitor of the EGFR family, is finely tuned by the phosphorylation state of Mig6. Mig6 inhibits EGFR by blocking the C- lobe asymmetric dimer interface. After Mig6 is phosphorylated by tyrosine kinases, its inhibitory potency is compromised, suggesting a potential strategy for cancer cells to escape the negative regulation of activated EGFR by Mig6. The EGFR kinase domain is depicted as blue, and segment 1 + 2 of Mig6 is depicted in purple. Figure from Wang et al, 2013.

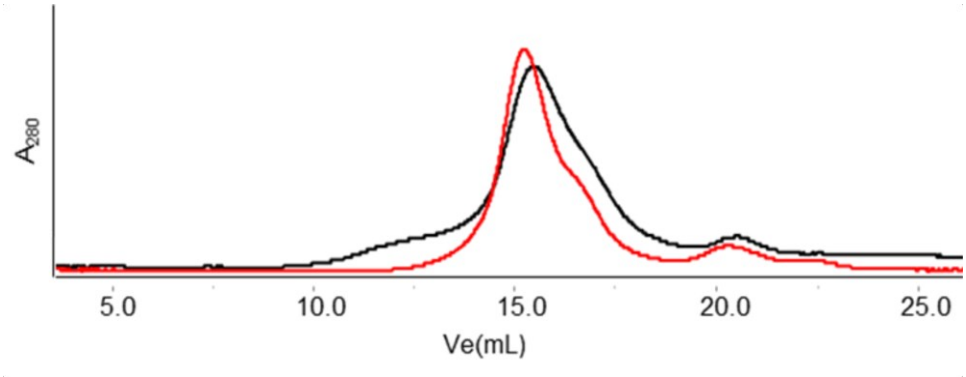


Figure 1.5 Packaging of Smoothened into MSP1E3D1 nanodiscs with DMPC. Smoothened packaged nanodiscs (red trace) elute at a higher molecular weight than empty nanodiscs (black trace) on a Superose 6 column (GE).

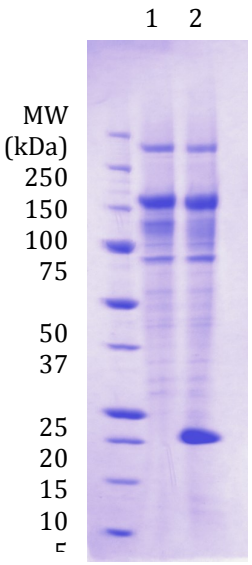


Figure 1.6 Packaging of IGF1R into MSP1D1 nanodiscs with DMPC. IGF1R (lane 1) survives packaging into nanodiscs after an overnight incubation at 4^{o}C (lane 2). The additional band around 24kDa corresponds to the MSP1D1 protein(Ritchie et al., 2009).

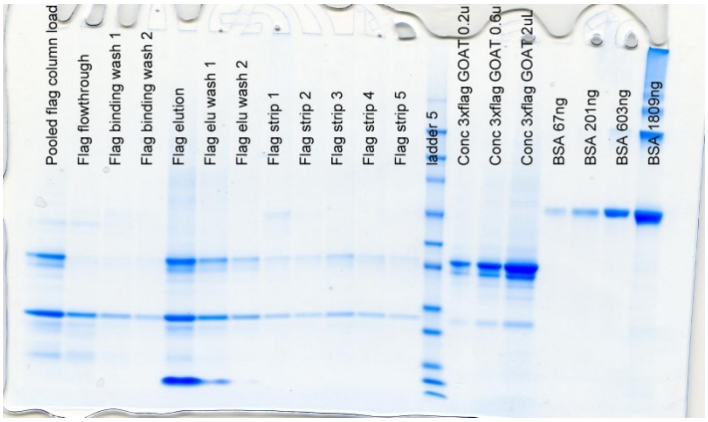


Figure 1.7 Packaging of GOAT into MSP1E3D1 nanodiscs with DMPC. Purification of FLAG-tagged GOAT nanodiscs is seen in elution from a FLAG affinity column. Lanes are as labelled, with the highest molecular weight band corresponding to BSA, then to GOAT, then to MSP1E3D1, then to the FLAG peptide used for elution. Figure prepared by Martin Taylor.

Chapter 2. Using Fab-fusion Proteins to Isolate Heterodimers

Acknowledgements

Min-Sung Kim and Kwangsoo Kim designed the Fab fusion constructs described below, and they and Jennifer Kavran were critical in the preparation and troubleshooting process. Nicholas Bessman performed all surface plasmon resonance (SPR) experiments.

Abstract

Cell surface receptors frequently form or function as multimers within a cell membrane but fail to multimerize when soluble fragments or detergent-solubilized forms of the receptor are studied. A common approach to overcome this problem is to fuse receptors or receptor fragments to dimeric fusion partners. We describe here use of the heavy and light chain components of the antigen-binding fragment (Fab) of an antibody to enforce heterodimerization of the extracellular regions of the Epidermal Growth Factor Receptor (EGFR/ErbB) family, which naturally form functional heterodimers between each of the four human EGFR homologs. We show that fusion of different ErbB extracellular regions to the two chains of an F_{ab} results in formation of functional heterodimers and that purification of these heterodimers using an affinity tag affixed to the heavy chain allows purification of heterodimers from light chain-mediated homodimers. Furthermore, use of an antibody directed against an epitope near the C-termini of ErbB extracellular regions enforces a rigid structural relationship between the Fab and the ErbB extracellular regions, which may prove beneficial for crystallization studies.

Introduction

A prerequisite for many biophysical and structural studies is isolation of a targeted protein or protein complex in a conformation of interest. This task can become difficult when the protein or proteins of interest normally form complexes at the cell membrane but fail to interact when solubilized or isolated as active fragments. A longstanding approach to this problem has been to enforce dimerization by expressing proteins fused to dimerizing partners such as the glycophorin A transmembrane domains(Engelman, Steitz, & Goldman, 1986), homodimeric leucine zippers(Landschulz, Johnson, & McKnight, 1988), heterodimeric leucine zippers (O'Shea, Lumb, & Kim, 1993), and the constant (F_c) regions of antibodies (Capon et al., 1989) (Muraki & Honda, 2010)(Yu et al., 2013)(Dickgiesser et al., 2015). Full heavy chain heterodimeric proteins have also been designed using "knobs-into-holes" engineering(Ridgway, Presta, & Carter, 1996). We describe here an approach to form heterodimers of targeted proteins using the heterodimeric antigen binding fragment (Fab) of antibodies.

We developed this system for application to the extracellular regions of member of the epidermal growth factor receptor (EGFR/ErbB1) family of receptor tyrosine kinases. Fc fusions have been used to study many facets of their EGFR homodimer activation(Jones, Akita, & Sliwkowski, 1999)(Burgess et al., 2008)(Bessman, Bagchi, Ferguson, & Lemmon, 2014), but all possible heterodimers of the 4 human ErbB homologs (EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4) appear to form and possess unique signaling properties (Holbro & Hynes, 2004). Dysregulation of these proteins is implicated in a variety of cancers, and many are the target of monoclonal antibody therapies and small molecule inhibitors. ErbB2 is overexpressed in

approximately 20% of breast cancers and is also implicated in cancers of the colon and lung(Holbro & Hynes, 2004), and heterodimerization with ErbB2 increases the affinity of its partners for their ligands(Jones et al., 1999), decreases receptor internalization(Tzahar et al., 1996), and ErbB2/ErbB3 heterodimers escape ErbB2 targeted therapy(Sergina et al., 2007) and are considered the most oncogenic pairing within this family(Holbro & Hynes, 2004). Although ErbB2 heterodimerization has clear functional and clinical implications, ErbB2-containing heterodimers must differ structurally from other ErbB family hetero- and homodimers(Alvarado, Klein, & Lemmon, 2010)(Liu et al., 2012), but the precise nature of these structural differences and how they influence function remain unclear. The kinase domains of these four homologs all adopt canonical kinase folds although the ErbB3 kinase domain lacks functional kinase activity (Zhang et al., 2006)(Holbro & Hynes, 2004), but isolated ECDs of ErbB2 and other ErbB homologs fail to dimerize in solutions up to concentrations as high as 0.1mM(Ferguson, Darling, Mohan, Macatee, & Lemmon, 2000). As numerous studies indicate highly active ErbB2-containing ErbB heterodimers, we designed an Fab-driven heterodimerization system to allow isolation and study of ErbB2/ErbB3 extracellular domain heterodimers.

Results and Discussion

Figure 1 diagrams our fusion scheme, where two ErbB extracellular regions (referred to hereafter as Protein A and Protein B to reflect the generality of this approach) are fused to either the heavy (IgG) or light chains (IgK) of an Fab. The IgG and IgK fusion proteins are then co-transfected into mammalian cells where they were secreted together and are subsequently purified. We used Fab 1479, an anti-ErbB4 antibody which is being developed as a potential breast cancer treatment(Hollmén, Määttä, Bald,

Sliwkowski, & Elenius, 2009), although many Fabs could be used. The 1479 antibody was chosen this case because it is known to bind to an epitope near the C-terminus of the ErbB4 extracellular region that can be transplanted into other ErbBs. By binding to this epitope, the Fab fusion partner will thus adopt a relatively rigid orientation relative to the ErbB heterodimer, which may enhance the success of attempts to crystallize expressed heterodimers. We also mutated the cysteine residues that form a disulfide bond between the IgG-IgK chains to allow visualization of dimer subunits when using nonreducing SDS PAGE. Flexible glycine-serine linkers(Ladurner & Fersht, 1997) were inserted between each protein of interest and either the heavy or light chain of Fab 1479. The length of the linkers were determined by modeling the distance between the ErbB C-termini and Fab N-termini based on crystal structures of 1479 bound to ErbB4(Hollmén et al., 2009) (Liu et al) and an ErbB4 homodimer(Liu et al., 2012) (Liu et al). As noted, we transplanted the 1479 epitope to the C-terminus of Protein 1 (in this case, the ErbB3 extracellular domain) to ensure a rigid association between these partners for subsequent crystallization trials. We used surface plasmon resonance (SPR) to see that our linkers, as designed, did not inhibit ligand binding. In fact, binding of ErbB2/ErbB3 heterodimers to the ligand Neuregulin was tighter than binding of the ErbB3 extracellular region alone to Neuregulin (191nM vs. 267nM). The affinity of expressed ErbB2/ErbB3 heterodimers for ligand was not significantly changed with longer linker length (191nM vs. 178nM), which implies that binding of ErbB2/ErbB3 heterodimers to Neuregulin was not constrained by glycine-serine linkers that are too short. Testing heterodimer activity as a function of linker length provides a general approach to ensuring optimal linker length depending on the system being studied.

Figure 2A depicts the fusion partner scheme that ultimately produced the highest yields of active, purified heterodimers. Both expressed chains had human growth hormone (hGH) fused to their N-terminus of a protein to aid expression in mammalian cell culture as well as detection (Leahy, Dann, Longo, Perman, & Ramyar, 2000).. The cDNA for interleukin-2 (IL-2) was placed C-terminal to the hGH tag for the IgG fusion to add 16kDa in molecular weight and allow visualization of otherwise similarly sized subunits using nonreducing SDS PAGE. We observed the presence of IgK homodimers, which were removed by Ni-NTA purification specific for a tag on the heavy-chain subunit, as seen in Figure 2B. Fab region heavy chains do not productively homodimerize(Schoonjans et al., 2000) and we see no evidence of IgG-Fab homodimers in our system (Figure 2B). IgG and IgK fusions co-migrate across affinity, anion exchange, and gel filtration chromatography at 1:1 stoichiometry, and can be effectively deglycosylated with PNGaseF. The fusion partners are also effectively removed with PreScission/3C protease (Figure 2C). Final yields for ErbB2-containing heterodimers were approximately 100 – 200ug/L of mammalian cells, and purified heterodimers elute as a monodisperse peak by gel filtration. Sufficient amounts of ErbB2/ErbB3 heterodimers were purified for X-ray crystallography trials, small angle x-ray scattering, and electron microscopy.

This Fab-driven heterodimerization scheme is easily adaptable to other proteins, and the epitope recognized by the Fab could be omitted or included as fits the experimental application. Fab-driven heterodimers expand on previously successful Fc fusions and represent a useful way to isolate soluble heterodimers of interest.

Materials and Methods

Cloning

The vectors developed for IgG and IgK fusion protein purification are pSGHP0 and pHSP0, respectively. pSGHP0 is modified from pSGHV0, described previously(Leahy et al., 2000), to include the cDNA for IL-2 3' to the gene for hGH and 5' to an octahistidine tag, followed by a 3C Protease/PreScission cut site. pHSP0 is similarly modified, with a streptactin binding protein (SBP) tag in place of IL-2. XbaI and NotI-HF (NEB) were used to introduce IgG and IgK fusion proteins into both vectors.

Mammalian cell transfection

HEK293 and GnTi-, a cell line useful for crystallization trials because all sites that are normally subject to N-glycosylation are glycosylated but not fully processed due to lack of N-acetylglucosaminyltransferase I(Chang et al., 2007), cells were maintained in suspension as described previously(Longo, Kavran, Kim, & Leahy, 2013) Mammalian cells were transiently transfected with high quality (OD260/280 = 1.88 – 1.92; OD260/230 = 2.1 – 2.2) purified DNA and polyethylene imine (PEI) at a 1:3 ratio, with 200ug of heterodimer vector introduced per liter of mammalian cells. Optimum co-transfection ratios of each IgG and IgK fusion were determined in small scale adherent culture before purification in large scale suspension. ErbB2/ErbB3 heterodimers were produced using 100ug of each construct per liter of cells, and equal DNA co-transfection gave optimum expression levels of all heterodimer constructs tested. 24 hours later transiently transfected cells were diluted 1:3 and grown in hybridoma media supplemented with 0.5% FBS for three days before heterodimer purification.

Heterodimer purification

Media from transiently transfected mammalian cells was spun at 1000g for five minutes then sterile filtered (0.22μm). Sodium azide was added to 1mM in filtered media. All subsequent purification steps were performed at 4ºC. A Centramate™ tangential flow filtration cassette (Pall) was used to concentrate media and buffer exchange into buffer A (20mM Tris 8, 1M NaCl). Five milliliters of Ni-NTA were allowed to bind in batch with concentrated heterodimers overnight. This solution was applied to a gravity column so that the Ni-NTA resin could be washed four times with 50mL of buffer A supplemented with 1) 1M urea, 2) 0.75% TX-100, 3) 1M Urea, and 4) 0.75% TX-100. These washes were followed by a 100mL wash of buffer A and elution with 60mL of buffer A with 250mM imidizaole. Nickel eluate was diluted to 1L in buffer A and applied to 5mL of freshly equilibrated Ni-NTA resin to batch bind overnight. This second batch of resin was applied to a gravity column and washed with 300mL of buffer A. Five milliliter fractions of heterodimers were eluted in 60mL of buffer A with 250mM imidazole. Fractions containing heterodimers were detagged and deglycosylated, as appropriate, with excess 3C Protease/PreScission and PNGaseF, respectively. Samples were dialyzed against four liters of 20mM Tris 7, 150mM NaCl, 1mM EDTA for two days or until cleavage was complete. Afterwards, samples were dialyzed against four liters of buffer A for at least one hour before application to a 5mL Ni Hitrap column (GE). The majority of untagged heterodimer did not bind this column and was collected in the flow-through, although some also came off with 250mM imidazole washes. Fractions containing heterodimers were dialyzed against four liters of 20mM Tris 8, 50mM NaCl overnight before application to a HiTrap Q column (GE). Heterodimers were eluted using buffer A, then

concentrated and loaded onto a G200 26/60 gel filtration column equilibrated in 5mM Tris 7.5, 150mM NaCl. Heterodimers were then concentrated as appropriate.

SPR

SPR binding experiments were performed using a Biacore 3000 instrument at 25°C. Neuregulin was immobilized in pH 5.0 acetate buffer, with ~100 response units (RUs) immobilized. ErbB3 extracellular domain or Fab-driven heterodimers were injected in serial 3-fold dilutions in 25 mM HEPES, pH 8.0, containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% Nonidet-P20. A flow rate of 5 µl/min was used for 10 minutes, which was sufficient to reach a stable RU equilibrium even at the lowest concentrations tested. Binding for each injection was calculated by subtracting the baseline RU reading from the RU response at 10 minutes post-injection. K_D values for binding of receptor variants to these surfaces were determined by fitting the equilibrium responses over a range of concentrations to binding models in GraphPad Prism 5.0. For ErbB3 extracellular domain binding to the neuregulin sensor chip, the data were adequately fit by a single-site Langmuir binding equation. For heterodimeric variants, better fits were achieved with a binding model specifying one specific binding site plus a non-saturable, weak, non-specific binding component. Between injections, surfaces were regenerated using 1 minute injections of 10 mM sodium acetate, pH 5.0, containing 1 M sodium chloride. Multiple rounds of regeneration did not impair receptor binding to the neuregulin surface.

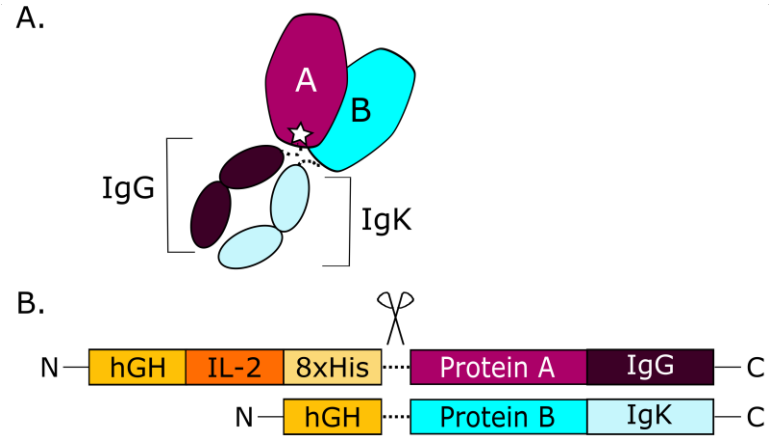


Figure 2.1 Fab-driven heterodimers. A) Cartoon representation of Fab-driven heterodimers, where the heavy chain (IgG) of Fab 1479 is fused by a flexible glycine-serine linker (dotted line) to a protein of interest, hereafter referred to as Protein 1. The epitope recognized by Fab 1479 is denoted by a white star, and is introduced to the C-terminus of Protein 1. This creates a rigid association between the IgG and Protein 1 to aid crystallization efforts. The light chain (IgK) of 1479 is similarly fused with a flexible glycine-serine linker to Protein 2.

B) Diagram representation of Fab-driven heterodimer proteins to be co-transfected in mammalian cells and purified as in Figure 2. The gene for human growth hormone (hGH) is introduced to the N-terminus of Fab fusion proteins as previously described (Leahy et al., 2000) to increase expression in mammalian cells. Protein 1 and Protein 2 designed for pilot studies (ErbB3-IgG and ErbB2-IgK) are of nearly identical molecular weight, and so the cDNA for interleukin-2 (IL-2) was introduced to enable easy differentiation of each fusion partner by SDS-PAGE. An octahistidine tag was added to only the IgG fusion partner, as IgG Fab homodimers are not a contaminant of concern(Schoonjans et al., 2000). A 3C Protease cleavage site allows for efficient tag removal after Fab-driven heterodimer isolation.

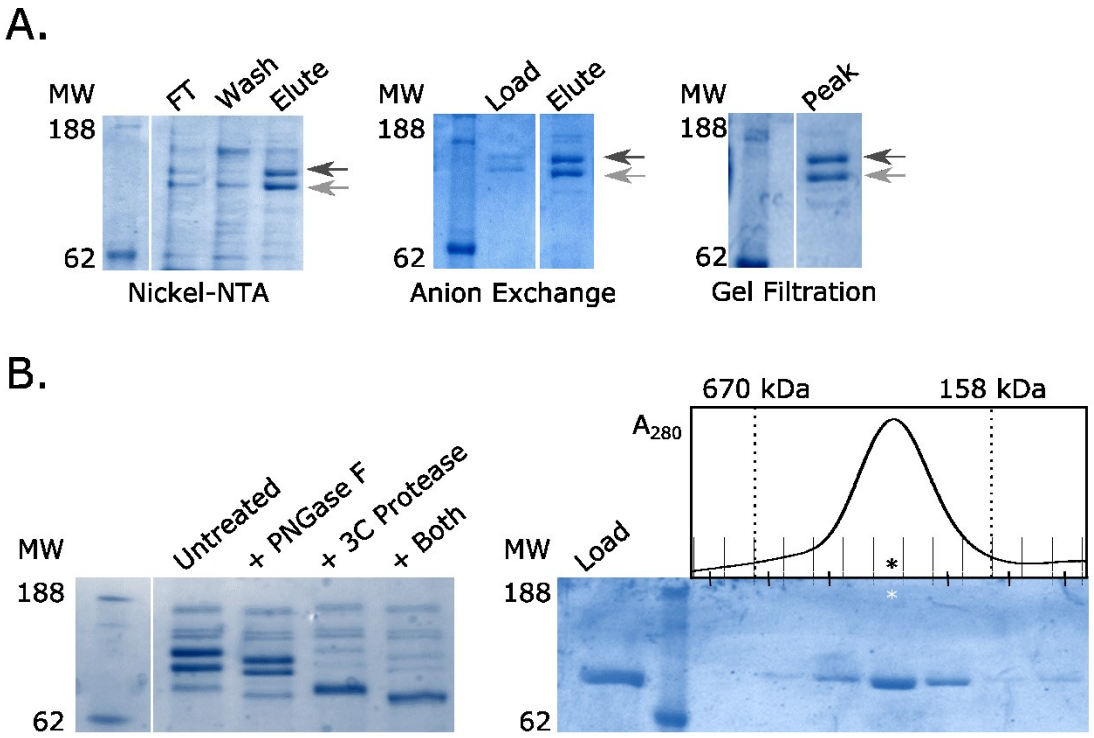


Figure 2.2 Purification of Fab-driven heterodimers. A) IgG and IgK fusion proteins co-migrate at stoichiometric levels over affinity purification, anion exchange, and gel filtration chromatography. The bands corresponding to IgG and IgK fusions are shown by dark and light gray arrows, respectively. All gels shown are representative of multiple heterodimer purifications. Contaminating IgK homodimers are removed through Ni-NTA purification, as seen in the flow-through and wash fractions.

B) I IgG and IgK fusion proteins are effectively deglycosylated and detagged by PNGaseF and 3C Protease treatment, respectively. Purified Fab-driven heterodimers elute as a monodisperse peak over gel filtration. Shown here is a representative gel filtration trace in the presence of ligand and an additional therapeutic antibody. Final yields were 100 – 200 μg per liter of mammalian cells.

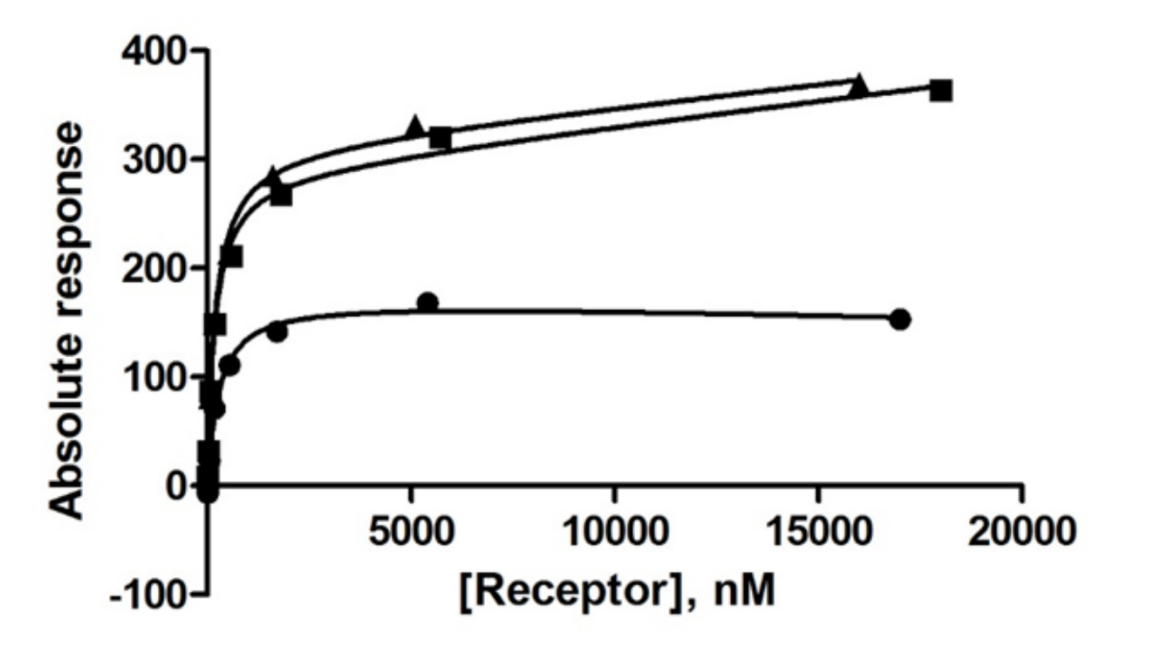


Figure 2.3. Binding of Fab-driven heterodimers to neuregulin. SPR data are shown for soluble ErbB3 extracellular domain (●), Fab-driven ErbB2/3 dimers as originally designed (▲), and ErbB2/3 dimers with longer glycine-serine linkers (■). Curves were fit with the assumption that one site bound ligand specifically, while another site bound non-specifically (as only one competent binding site is present within each heterodimer and sErbB3). The K_d for each of these dimers for neuregulin is 267nM, 191nM, and 178nM, respectively. Linker length did not significantly alter ligand binding, demonstrating that linker length was not constraining in our system. Experiment performed by Nicholas Bessman.

Chapter 3. Heterodimer Crystallography, SAXS, and EM

Acknowledgements

I was trained by Leahy laboratory members, principally Min-Sung Kim and Jennifer Kavran, in protein purification and crystallography. I was trained by Nick Bessman and Sandra Gabelli to collect and analyze SAXS data. I was trained to analyze EM data by Nick Bessman, Dewight Williams, and Ernesto Arias-Palomo. Dewight Williams set all EM grids, and Ernesto Arias-Palomo generated and compared all 2-D projections of ErbB2/3 heterodimer models to EM class averages.

Abstract

The "deaf and the dumb" members of the ErbB family of receptor tyrosine kinases, namely ErbB2 which does not respond to ligand and ErbB3 which has an essentially inactive kinase domain(Citri, Skaria, & Yarden, 2003), are considered the most oncogenic heterodimer pair within the ErbB family(Holbro & Hynes, 2004). Despite the availability of high resolution structures of the ErbB extracellular and intracellular domains, it is still unclear how heterodimerization with ErbB2 evokes such exaggerated responses. Although the extracellular domains of liganded ErbB's look similar to the ErbB2 ECD, a 30^{o} domain rotation prevents ErbB2-containing heterodimers from adopting the same conformation as other ErbB ECD homodimers. (Figure 3.1). The Fab-heterodimerization scheme described in Chapter 2 was used to purify ErbB2/3 heterodimers as well as other physiologically relevant ErbB heterodimers for structural studies. Unfortunately crystallization trials were unsuccessful, as were small angle X-ray scattering (SAXS) and negative stain electron microscopy (EM) analyses of ErbB2/3 heterodimers. Initial designs for studying the energetics of ligand binding to

various EGFR-containing heterodimers by isothermal titration calorimetry (ITC) were hindered by insufficient protein yields. These preliminary studies are described below.

Introduction

The Fab-driven heterodimerization scheme described in Chapter 2 was designed specifically for the crystallization and subsequent structural determination of an ErbB2-containing heterodimer. The ErbB2/3 heterodimer is considered the most relevant to human cancers(Holbro & Hynes, 2004) and current high resolution structures do not resolve how ErbB2-containing heterodimers mediate their exaggerated effects on cell growth and proliferation (Figure 3.1). Cursory surface plasmon resonance experiments showed that the binding affinity of ErbB2/3 heterodimers for neuregulin was tighter than for the soluble ErbB3 ECD alone, as predicted by previous work(Jones et al., 1999), and was not significantly different if the glycine-serine linkers between ErbB ECDs and their IgK/IgG fusion partners were lengthened (Figure 2.3, Table 3.1). This led us to believe that there was nothing inherently amiss about our Fab-driven ErbB family heterodimers and that crystallization trials were performed with proteins that, if crystallized, would be useful approximations of ErbB family heterodimers *in vivo*.

Exhaustive crystallization trials were conducted for ErbB2/3 heterodimers in the presence and absence of ligands and relevant therapeutic antibodies. Although small crystals were obtained in the presence of neuregulin (Figure 3.3), we were unable to optimize them to diffract to a sufficient resolution for structural determination. Additional efforts, summarized in Table 3.2, included setting trays with ErbB2/ EGFR and with ErbB2/3 heterodimers with longer glycine-serine linkers. In total, more than 3,600 and

1,100 conditions were tested for crystallization of ErbB2/3 and ErbB2/EGFR heterodimers, respectively.

Putative ErbB2/3 microcrystals were initially screened by Tom Murray for use with a microbeam synchrotron source, but additional studies were not pursued as they did not detectably diffract X-rays. ErbB2/4 and asymmetric EGFR heterodimers, where one EGFR can bind ligand and the other EGFR cannot, were similarly prepared at small scale with later crystallization trials in mind. These were not ultimately pursued in large scale because the ErbB2/4 heterodimer is less physiologically relevant than the ErbB2/3 and ErbB2/EGFR dimers(Holbro & Hynes, 2004), and initial purification efforts of asymmetric EGFR produced low yields (~20ug/L. Focus shifted towards using small angle X-ray scattering (SAXS) and negative stain electron microscopy (EM) to capitalize on our purified ErbB2/3.

We attempted to use SAXS and EM to determine whether the ErbB3 ECD favors the tethered or extended conformation in the absence of ligand, as illustrated in Figure 3.4. We pursued this as a logical follow-up to work done by the Lemmon laboratory, where Fc-driven EGFR homodimers were purified and studied with these techniques(Bessman et al., 2014). In this report, despite predicted particle dimensions nearing the detection limit for negative stain EM and SAXS (Figure 3.4), a clear difference was visible between well-ordered ligated EGFR homodimers compared to unliganded dimers. We thus predicted that we could obtain similar resolution data to address our question.

Unfortunately, we were unsuccessful. The differences in SAXS envelopes from the ErbB3 extended and tethered models were very small (Table 3.3) and no discernible

difference in model fit can be seen from final SAXS envelopes produced using data from the SAXS source at the University of Pennsylvania (Rigaku S-MAX3000 pinhole camera system with a Rigaku 007HF rotating anode source and a Rigaku 300 mm wire grid ASM DTR 200 detector) (Figure 3.5). Although the envelopes obtained are of the right size and contain the predicted curvature expected from 1479 Fab binding, data collected in the presence or absence of ligand were essentially indistinguishable.

This observation may be because the ErbB3 ECD is extended in the absence of ligand, but we were unable to resolve this possibility. We attempted to obtain a control envelope where one partner was extended (ErbB2) and the other were tethered by binding the Fab of cetuximab (Erbitux©), an antibody that keeps the EGFR ECD in a tethered conformation, to ErbB2/EGFR homodimers. However, we did not see cetuximab binding to these heterodimers and thus could not compare our data to this reference.

Similarly, negative stain EM class averages revealed particles of roughly the expected size and shape but provided inconclusive information. 2-D projections were generated from both the ErbB3 extended and tethered models and compared to class averages. To reduce bias during model fitting, all 2-D projections were compared to all class averages in the presence and absence of neuregulin. The final results of these comparisons are summarized in Table 3.4, and a representative comparison of class averages to 2-D projections is shown in Figure 3.6. These comparisons were performed by Ernesto Arias-Palomo, a member of the lab of James Berger who has extensive EM experience.

If our data could accurately distinguish whether ErbB3 is extended or tethered within our ErbB2/3 heterodimers, we would expect our samples collected in an excess of

ligand to adopt almost exclusively an extended model conformation. However, our data do not show clearly show this conformation, and thus we cannot draw any conclusions from this work at this time.

Bessman et al. also used a GE MicroCal ITC200 to perform isothermal titration calorimetry (ITC) to study the relationship between ligand binding and dimerization within tethered EGFR homodimer. A logical continuation was to expand this work to cover EGFR family heterodimers, where the following titrations would be performed:

1. EGFR/EGFR with 1479 epitope + EGF
2. EGFR/EGFR without epitope + EGF
3. Asymmetric EGFR + EGF
4. EGFR/ErbB2 + EGF
5. ErbB3/ErbB2 + neuregulin
6. ErbB3 + neuregulin

Titrations 1 and 2 would have shown if the rigid association of Fab-driven heterodimers containing the 1479 epitope affected ligand binding and if we could reproduce the work from Bessman et al. Titration 3 would have mimicked the singly-ligated *Drosophila* EGFR homodimer. Titration 4 would have demonstrated the effects of ErbB2 on ligand binding, Titration 5 would have compared ErbB2's effects of EGFR to those on ErbB3, and the binding of ErbB3 would have served as a control.

In their work, they used roughly 10μM of protein in 200μL of a reaction cell per titration. Under similar reaction conditions, approximately 0.5mg of purified Fab-driven heterodimer would be needed per titration. While this is a large amount of protein given optimum yields of 200μg/L of mammalian cells for ErbB2/3 purification, it would have

been feasible if final yields of other EGFR family heterodimers were similar. However, the necessary heterodimers were not highly expressed in either mammalian or insect cell lines. Insufficient final yields of EGFR homodimers containing the 1479 epitope prevented this necessary titration from being completed and thus negated the utility of any further titrations.

Materials and Methods

Crystallization Trials

Fab-driven heterodimers were purified as described in Chapter 2 and Appendix II, with ligands and relevant therapeutic antibodies added as appropriate, and concentrated to 1 – 5mg/mL. A Mosquito liquid handling robot (TTP Labtech) was used for 96-well screening using standard crystallization kits. Promising conditions were followed up in 24-well plates.

Small Angle X-ray Scattering (SAXS)

X-ray scattering data were collected at three concentrations of ErbB2/3 heterodimer (typically 2, 4, and 6mg/mL; or 1, 3, and 5mg/mL) and radially averaged and initially processed using SAXSgui. The ATSAS program suite was used to further process SAXS data(Svergun & Koch, 2003). PRIMUS was used to assess data quality and determine radius of gyration (R_g) values(Konarev, Volkov, Sokolova, Koch, & Svergun, 2003), GNOM was used to determine maximum interatomic distance (D_{max}) values from P(r) curves(Svergun, 1992), DAMMIN(Svergun, 1999) and DAMMIF(Franke & Svergun, 2009) were used to generate models from SAXS data that were aligned with DAMAVER(Volkov & Svergun, 2003). Expected R_g and D_{max} values

for ErbB3 extended and tethered heterodimer models were generated in Crysol(Barberato, Koch, Molecular, & Outstation, 1995).

Negative Stain Electron Microscopy (EM)

Staining conditions were modeled after those used in Bessman et al. and grids were set by Dewight Williams, Director of Electron Microscopy Resources at the Biochemistry and Biophysics Department at University of Pennsylvania. Raw grid images were visualized using Gatan Digital Micrograph (Gatan). Particle picking was done manually using the EMAN2 software package(Tang et al., 2007) and class averages were generated by Dewight Williams using SPIDER(Shaikh et al., 2008). 2-D projections were generated in EMAN2 from volume models created in Chimera(Pettersen et al., 2004) and compared to class averages using standard procedures by Ernesto Arias-Palomo.

Results

Crystallization Trials

Exhaustive crystallization trials produced small (<60μm) crystals of ErbB2/3 heterodimers in the presence of neuregulin (Figure 3.3) that could not be improved and did not diffract X-rays. Useful crystals were not obtained for ErbB2/3 heterodimers in the presence or absence of relevant therapeutic antibodies, nor were crystals obtained for ErbB2/EGFR heterodimers (Table 3.2).

Small Angle X-ray Scattering (SAXS)

Although final SAXS envelopes were roughly consistent with predicted models for ErbB2/3 heterodimers, no conclusive statement about ErbB3 ECD domain dynamics

within the context of a heterodimer can be made (Figure 3.5). This is consistent regardless of light source, and thus roughly the quality of data, used.

Negative Stain Electron Microscopy (EM)

Similar to the final SAXS envelopes, select negative stain class averages roughly correspond to the predicted size and shape of ErbB2/3 heterodimers but do not provide interpretable structural information (Figure 3.6).

Proposed Isothermal Titration Calorimetry

The heterodimers needed for titrations 1 – 6 were not expressed highly from mammalian nor insect cell lines. EGFR homodimers containing the 1479 epitope could not be purified at greater than 10ug/L final yields. Thus, these experiments could not be performed.

Discussion

Although our biophysical and structural studies of ErbB2/3 heterodimers could not be completed (crystallization, ITC) or were inconclusive (SAXS, EM), the heterodimerization method described in Chapter 2 may be broadly applicable to other systems. It is possible that further negative stain or Cryo-EM may address whether the ErbB3 ECD is principally extended or tethered within an ErbB2/3 heterodimer.

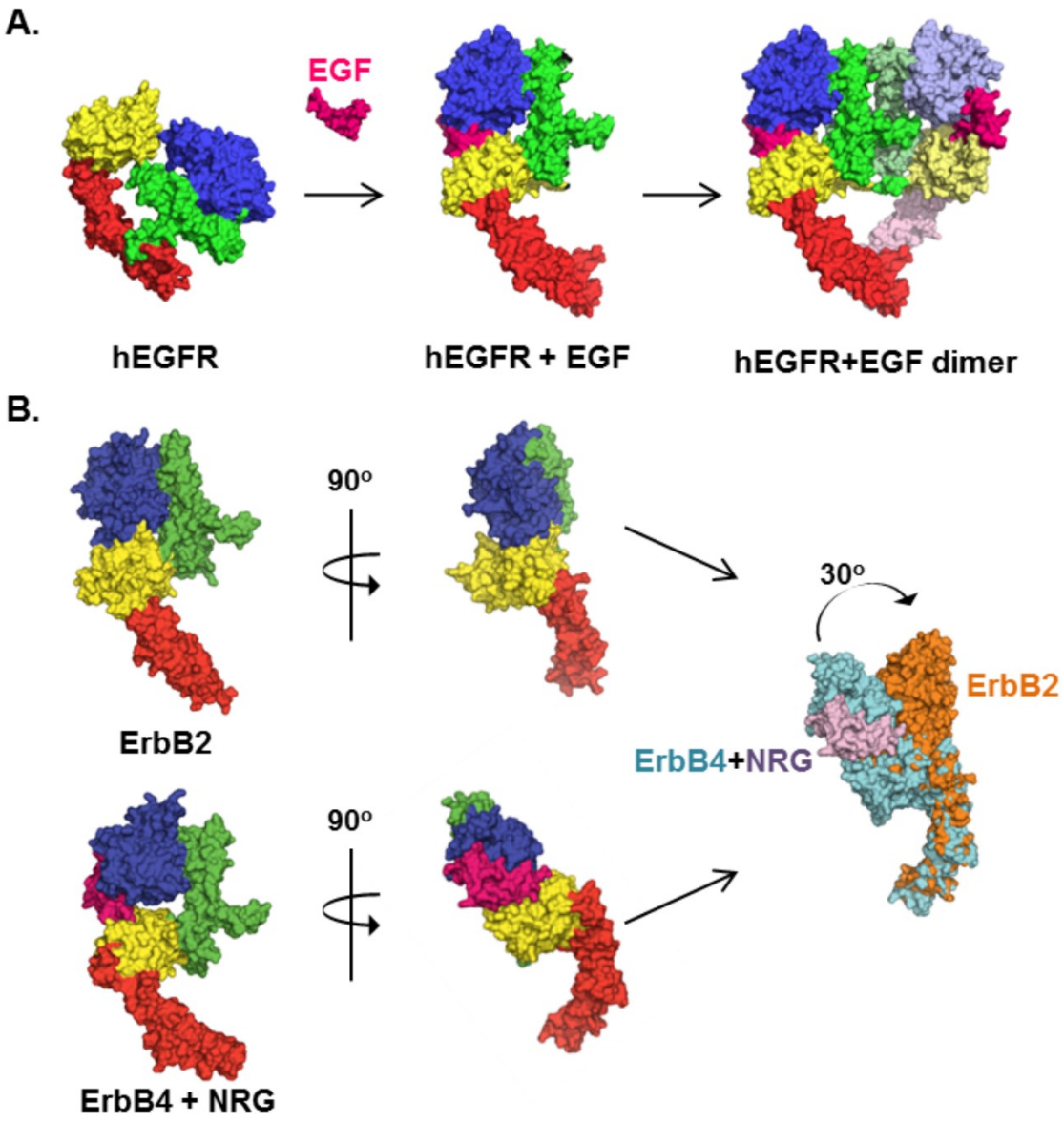


Figure 3.1 Key structural differences must be accommodated in ErbB2-containing heterodimers. A) Ligand binding induces ECD conformational change in hEGFR, ErbB3, and ErbB4. C) ErbB2 resembles ErbB4 bound to neuregulin (NRG), but has ~30° relative domain rotation. Structures from (Burgess et al., 2008), (Liu et al., 2012), and (Cho et al., 2003).

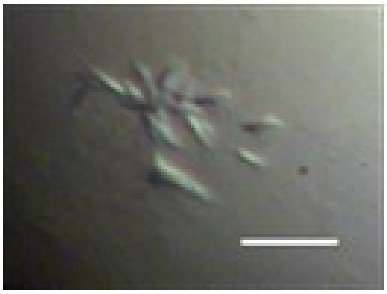


Figure 3.2 ErbB2/3 Crystals. Optimum ErbB2/3 heterodimer crystals formed in the presence of neuregulin. Despite extensive efforts crystals could not be improved. Scale bar is 60μM.

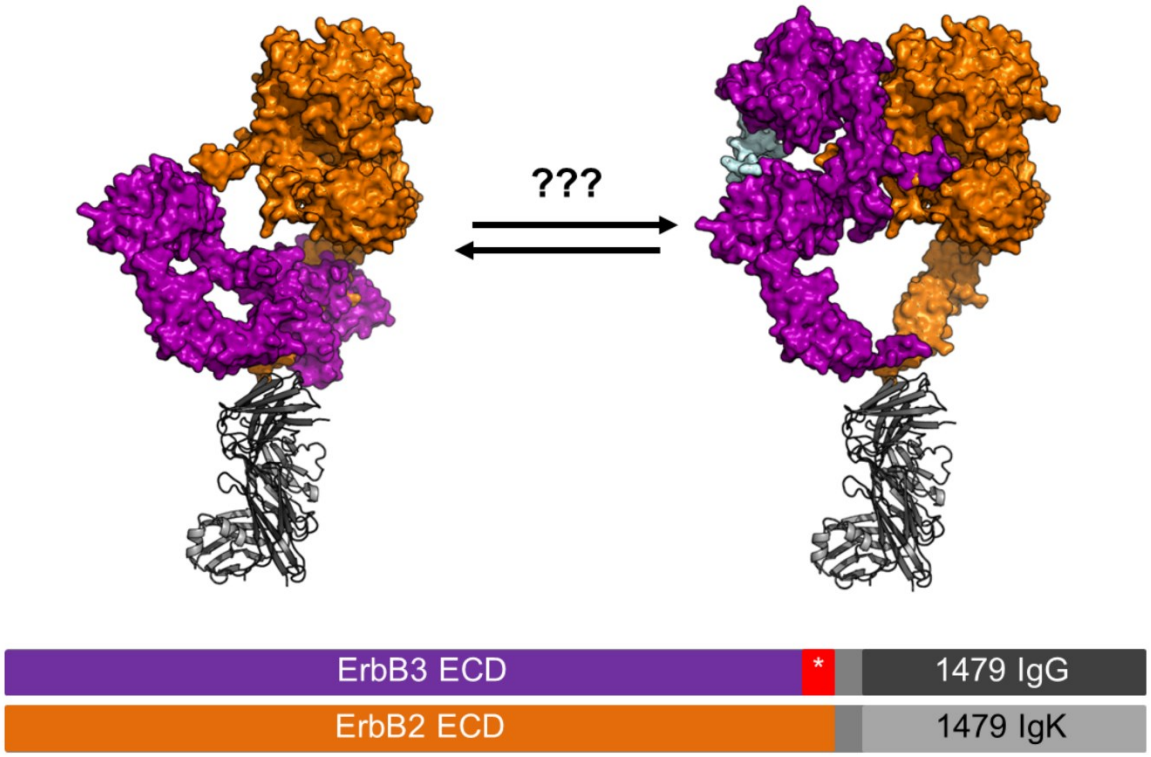


Figure 3.3 Model for SAXS and EM studies. After unsuccessful crystallization efforts, we pursued small angle x-ray scattering (SAXS) and negative stain electron microscopy (EM) to determine if the ErbB3 ECD favors a tethered or extended conformation within an ErbB2/3 heterodimer. The above is a representation of this research question. This structural model is based on the crystal structures of singly-ligated *Drosophila* EGFR(Alvarado et al., 2010) and the extracellular domains of ErbB3(Ferguson et al., 2003) and ErbB2(Cho et al., 2003).

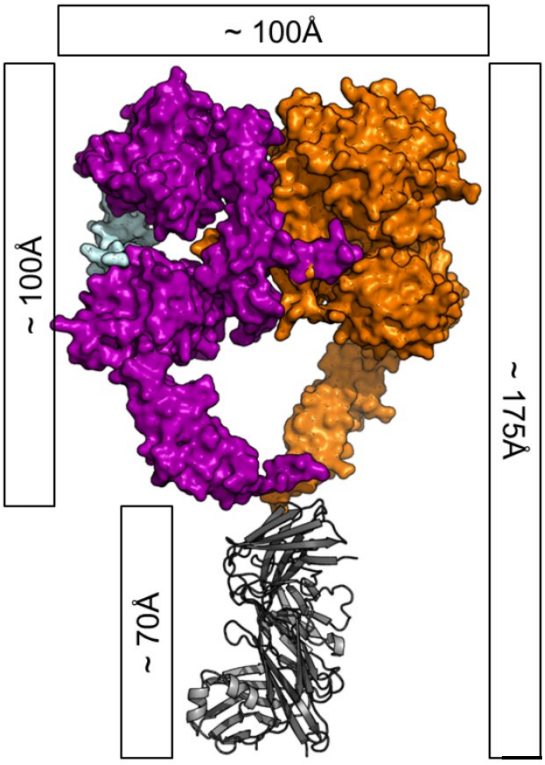


Figure 3.4 Predicted dimensions of an Fab-tethered ErbB2/3 heterodimer where ErbB3 is extended. This structural model is based on the crystal structures of singly-ligated *Drosophila* EGFR(Alvarado et al., 2010) and the extracellular domains of ErbB3(Ferguson et al., 2003) and ErbB2(Cho et al., 2003).

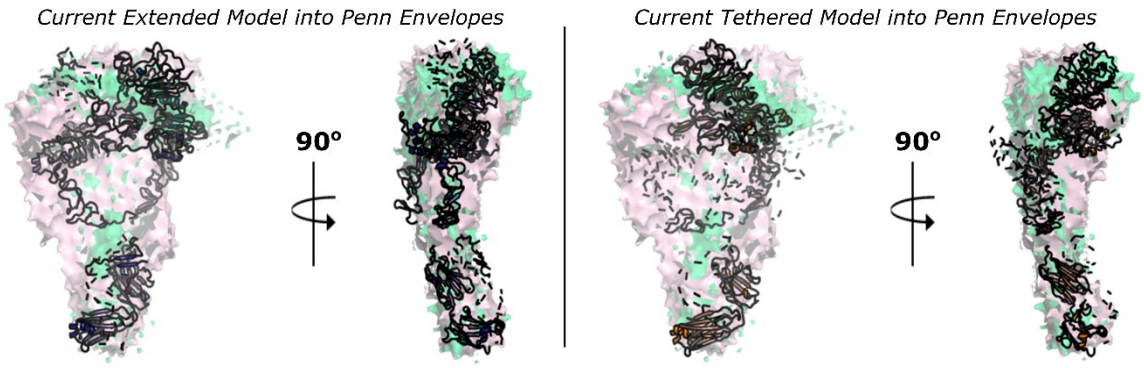


Figure 3.5 Final SAXS envelopes. These envelopes were generated from data obtained from the SAXS source at the University of Pennsylvania in the absence of ligand. Similar envelopes were obtained from the Rigaku BioSAXS-2000. Neither envelope conclusively fits either the ErbB3 extended or tethered models, consistent with SAXS parameters listed in Table 3.3.

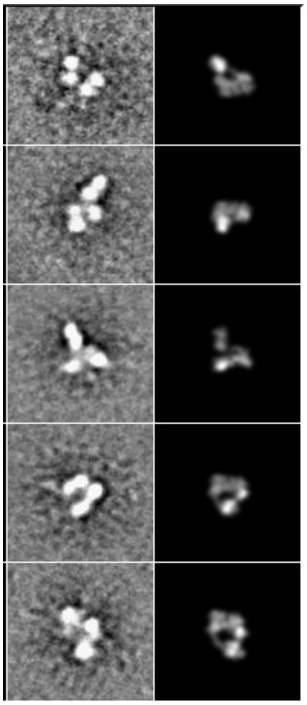


Figure 3.6 Representative EM class averages and 2-D model projections. Selected class averages from ErbB2/3 without ligand (left) vs 2-D projections of ErbB3 extended model (right). Similar class averages were seen in samples with ligand, and neither set of class averages agrees significantly with the ErbB3 extended or tethered models. Correlations are shown in Table 3.4.

	Binding Affinity for NRG		Binding Affinity from Jones et al, 1999
	05/06/2013	**03/15/2014**	
ErbB3 ECD	152nM	267nM	1 – 100nM
ErbB2 + 4 GS ErbB3 + 6 GS	59nM	191nM	<1nM (no linker)
ErbB2 + 6 GS ErbB3 + 8 GS	-	178nM	<1nM (no linker)

Table 3.1 Neuregulin binding to ErbB2/3 heterodimers by SPR. Assays performed by Nick Bessman on different dates, as indicated below. Binding affinity values from the Sliwkowski groups are included for comparison(Jones et al., 1999). Surface plasmon resonance (SPR) traces from 03/15/2014 are shown in Figure 3.2, results alluded to in Chapter 2.

Protein	Co-express small scale	Co-express large scale?	Purified?	Trays? +/- ligand? Abs?	Xtals?	Data?
H3/H2	Yes 2mg/L	Yes 2mg/L	Yes	Yes, both	Small w/ NRG	No
H3/H2 longer linker	Yes	Yes	Yes	not NRG and Trastuzumab	-	-
H1/H2	Yes 0.46mg/L	Yes 0.8mg/L	Yes	Yes, both	None	-
H4/H2	Yes 0.8mg/L	-	-	-	-	-
Asymm-etric hEGFR	Yes 4mg/L	Yes 0.65mg/L	Yes	-	-	-

Table 3.2 Summary of Crystallization Efforts. Greater than 3,600 crystallization conditions of antibody driven HER2/HER3 heterodimers, including combinations of all relevant therapeutic antibodies and ligands available to the Leahy laboratory, and screening of approximately 1,100 conditions for crystallization of HER2/EGFR heterodimers did not produce crystals that diffracted to a sufficient resolution for structural determination. Other heterodimers were attempted and were likewise unsuccessful.

Source	**Penn**		**Rigaku**	
	Alone	with NRG	Alone	with NRG
Rg	50 +/- 4.72 Å	65 +/- 13.3 Å	70 Å	60 Å
Dmax	200 Å	200 Å	250 Å	220 Å
Crysol Predicted				
	Tethered		Extended	
Rg	55 Å		60 Å	
Dmax	196 Å		196 Å	

Table 3.3 Summary of Small Angle X-Ray Scattering (SAXS) Parameters. Expected differences between envelopes where the ErbB3 extracellular domain is extended and tethered were small, and comparing data collected from the SAXS source at the University of Pennsylvania ("Penn") and from the Rigaku BioSAXS-2000 ("Rigaku") could not resolve if one model better fit our data. SAXS envelopes corresponding to this table are shown in Figure 3.4.

	Number of Class Averages Better Fit with each Model			
Samples	Extended ErbB3	Tethered ErbB3	Both Equally	Neither Fits
With NRG	9 /20	6 /20	3 /20	2 /20
Without NRG	9 /20	8 /20	3 /20	0 /20

Table 3.4 Summary of Electron Microscopy Efforts. Attempts to fit 2-D projections of the ErbB3 extended nor ErbB3 tethered ErbB2-containing heterodimer models (Figure 3.2) to class averages obtained from electron stain microscopy were unsuccessful. Sample class averages compared to 2-D projections are in Figure 3.5. EM grids set with Dewight Williams and 2D projections and comparisons courtesy of Ernesto Arias-Palomo.

Appendix I: Reagent Locations

1.1 DNA Catalog – List of Constructs
"Raines Box 1" also called Leahy Lab DNA Stock Box 14

Vectors	**Insert**	**Cut In With**	**Location**
pSGHRP0	-	-	14/Raines Box 1, 2
pHSP0	-	-	Raines Box 1, 3
pSGHV0	-	-	Raines Box 1, 4
pSSXm	-	-	Raines Box 1, 5
pCDNA 3.1	-	-	Raines Box 1, 6
pFastBac HBM	-	-	Raines Box 1, 7
Fab-driven Heterodimer Fusion Proteins			
Vector	**Insert**	**Cut In With**	**Location**
pSGHRP0	H1*G	"	Raines Box 1, 32
	H3*G	5' XbaI/3' NotI	Raines Box 1, 33
	H4G	"	Raines Box 1, 34
	EG	"	Raines Box 1, 35
	H3*G + 8 gs		Raines Box 1, 43
pHSP0	H2K	5' XbaI/3' NotI	Raines Box 1, 52
	LK	"	Raines Box 1, 53
	EK	"	Raines Box 1, 54
	H4G	"	Raines Box 1, 55
	H2K + 6 gs		Raines Box 1, 62
pSGHP1	H1*G	5' XbaI/3' NotI	Raines Box 1, 72
	H2K	"	Raines Box 1, 73
	H4G	"	Raines Box 1, 74-5
pSSX	EG	5' XbaI/3' NotI	Raines Box 1, 59
	H3wtG	"	Raines Box 1, 69
Ligands			
Vector	**Insert**	**Cut In With**	**Location**
pET32a	NRG1β	?	Raines Box 1, 17
	NRGβ56	?	Raines Box 1, 18
pT7HT	hEGF	?	Raines Box 1, 19
Other Proteins			
Vector	**Insert**	**Cut In With**	**Location**
pSSX	IGF1R	?	Raines Box 1, 79
pLEXm	WT tEGFR	5'NotI/3'XhoI	Raines Box 1, 82
pLEXm	delEA tEGFR	"	Raines Box 1, 83-4
pLEXm	L858R tEGFR	"	Raines Box 1, 85-6
pLEXm	FL EGFR with kinase mutant		Raines Box 1, 87-8
pMal3CBN	IL2 cDNA BALB/C	5' BamHI/3' NotI	Raines Box 1, 8
pMal3CBN	IL2 cDNA RF	5' BamHI/3' NotI	Raines Box 1, 9
???	*"ErbB4 Ab N-term"*	*?*	*Raines Box 1, 10*
MSP1D1	http://www.addgene.org/20061/		Raines Box 1, 92-93
MSP1E3D1	http://www.addgene.org/20066/		Raines Box 1, 94-95

Full information for MSP1D1/MSP1E3D1 available on Addgene

DNA Catalog – Sequence Information

Empty Vector: pHSP0

Derived from: pSGHV0 for mammalian expression
Tags introduced using: 5'XhoI/3'BamHI (relevant sites underlined)
Tags: hGH gene – SBP tag – 3C Protease cleavage site
MCS: Same as parent
Sequence:

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Insert: LK

Description: EGFR ECD with D355A, can't bind ligand + linker + Fab 1479 IgK

Italic: linker; Bold: IgK

Introduce Insert: 5'XbaI/3'NotI

Sequence:

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Vector + Insert: pHSP0 + LK

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Insert: EK

Description: EGFR ECD + linker + Fab 1479 IgK
Underline: linker; Bold: IgK
Introduce Insert: 5'XbaI/3'NotI
Sequence:

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Vector + Insert: pHSP0 + EK

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TTTGACAGCTTATCATCGAT

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pSGHRP0

Derived from: pSGHV0 for mammalian expression

Tags introduced using: 5'XhoI/3'BamHI (relevant sites underlined)

Tags: hGH gene – IL2 RF cDNA – 8x His tag – 3C Protease cleavage site

MCS: Same as parent

Sequence:

AAGCTTGGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGT
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Insert: H3*G

Description: ErbB3 ECD with Fab epitope + linker + Fab 1479 IgG
Underline: linker; Bold: IgG
Introduce Insert: 5'XbaI/3'NotI
Sequence:

GGATCCTCTAGAGCCACCATGTCCGAGGTGGGCAACTCTCAGGCAGTGTGTCCTGGGACTCTGAATGGCCTGAGTGTGACCGGCGATGCTGAGAACCAATACCA
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Vector + Insert: pSGHRP0 + H3*G

Sequence:

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Insert: H1*G

Description: EGFR ECD with Fab epitope + linker + Fab 1479 IgG
Underline: linker; Bold: IgG
Introduce Insert: 5'XbaI/3'NotI
Sequence:

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Vector + Insert: pSGHRP0 + H1*G

Sequence:

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Insert: H4G

Description: ErbB4 ECD with Fab epitope + linker + Fab 1479 IgG

*** note: Fab 1479 epitope from ErbB4, no need to mutate***

Underline: linker; Bold: IgG

Introduce Insert: 5’XbaI/3’NotI

Sequence:

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Vector + Insert: pSGHRP0 + H4G

Sequence:

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Insert: EG

Description: WT EGFR ECD + linker + Fab 1479 IgG

Underline: linker; Bold: IgG

Introduce Insert: 5’XbaI/3’NotI

Sequence:

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Vector + Insert: pSGHRP0 + EG

Description: WT EGFR ECD + linker + Fab 1479 IgG

Introduce Insert: 5’XbaI/3’NotI

Sequence:

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AAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGG
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CGTCTTCAAGAATTGGTCGACCAATTCTCATGTTTGACAGCTTATCATCGAT

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pSGHBP0

Never used for Fab-fusion purifications

Derived from: pSGHV0 for mammalian expression

*** Same as pSGHRP0 except using BALB/C IL2***

Tags introduced using: 5'XhoI/3'BamHI (relevant sites underlined)

Tags: hGH gene – IL2 RF cDNA – 8x His tag – 3C Protease cleavage site

MCS: Same as parent

Sequence:

AAGCTTGGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGT
GTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAA
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AGAATTGGTCGACCAATTCTCATGTTTGACAGCTTATCATCGAT

pSGHCSV0

Never used for Fab-fusion purifications

Derived from: pSGHV0 for mammalian expression

Tags introduced using: 5'XhoI/3'BamHI (relevant sites underlined)

Tags: hGH – TEV – MCS – TEV – SBP - stop

MCS: Same as parent

Sequence:

AAGCTTGGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGT
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TAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTGGTCGACCAATTCTCATGTTTGACAGCTTATCATCGAT

1.2 Glycerol Stocks of DNA

Location: Raines Glycerol Stock Box
All stocks made with DH10B E.Coli cells unless otherwise noted in relevant purification protocol. Vectors same as listed above or in relevant protocols unless otherwise noted.

WT tEGFR	-	-	-	-		-	IL2	-
-	NRGβ56	NRG1β	hEGF	-		-	IL2	-
-	-	-	-	-	-	-	-	3C Protease
pSGHB P0	-	-	-	-	-	-	-	-
pSGHR P0	-	pSGHR P0 + EG	pSGHR P0 + H1*G	-	pSGHR P0 + H3*G	pSGHR P0 + H4G	-	tEGFR L858R
pSGHC SV0	-	-	-	pSG HCSV0 + H2K	-	-	-	tEGFR delEA
pSGHV 0	-	-	-	pSGHP1 + H2K	-	-	-	-
pHSP0	-	-	pHSP0 + H1*G	pHSP0 + H2K	pHSP0 + H3*G	pHSP0 + H4G	pHSP0 + LK	pHSP0 + EK
-	-	-	-	-	-	-	-	-

1.3 Purified Protein Stocks

Assorted ErbB Family Ligands – Raines Box 2

Name	Concentration (year-mo-day)	Volume aliquots (uL)/color
His- NRG1β	0.28 mg/mL (130307)	1mL/red pen clear 1mL tube
NRG1β	~1mg/mL (130307)	1mL/black, clear 1mL tube
NRGβ56	9.48mg/mL (141113)	100µL/in blue cap 50mL tube
hEGF	1.7 mg/mL (150612)	120µL/in red cap 50mL tube
Herceptin Fab	0.35mg/mL (130911)	1mL/clear 1mL tube
Cetuximab Fab	3.7mg/mL (140627)	100µL/blue PCR tube, "C"

Membrane Scaffold Proteins/Chapter 1 Related – Raines Box 3

Name	Concentration	Volume aliquots (uL)/color
His- MSP1D1	1mg/mL	25µL/purple PCR tube
MSP1D1	1mg/mL	25µL/yellow PCR tube
His-MSP1E3D1	1mg/mL	250µL/orange PCR tube
MSP1E3D1	1mg/mL	250µL/blue PCR tube
WT tEGFR	100uM	~5uL/clear PCR tube
PreScission	3mg/mL	~1mL/clear 1mL tube

1.4 Cell Lines – Raines Liquid Nitrogen Stocks
Tower 11, Box 1
*** Note: I clone stable cells lines may all be currently contaminated with IGF1R WT, confirmed in 1I-2, 5I-2, and 6I-1.***

Key to I clones (continuation of McCabe Thesis, see for more detail)
6I: IGF1R (ECD) – cys – IGF1R (TM-ICD) | TM-ICD ~ 58.6kDa (895 – 1428)
5I: IGF1R (ECD) – cys – IR (TM-ICD) | TM-ICD ~46.8kDa (957 – 1382)
1I: IGF1R (ECD) – cys – EGFR (TM-ICD) | TM-ICD ~59.7kDa (643 – 1186

1 CHO-S p14 150817	2 CHO-S p14 150817	3 CHO-S p14 150817	4 CHO-S p14 150817	5 -	6 -	7 -	8 -	9 -
10 -	11 -	12 -	13 -	14 -	15 -	16 -	17 -	18 -
19 6E1, CHO-S p8 150813	20 6E1, CHO-S p8 150813	21 6E1, CHO-S p8 150813	22 -	23 -	24 -	25 -	26 -	27 -
28 6E5, CHO-S p8 150813	29 6E5, CHO-S p8 150813	30 6E5, CHO-S p8 150813	31 -	32 -	33 -	34 -	35 -	36 -
37 6E6, CHO-S p8 150813	38 6E6, CHO-S p8 150813	39 6E6, CHO-S p8 150813	40 -	41 -	42 -	43 -	44 -	45 -
46 -	47 -	48 -	49 -	50 -	51 -	52 -	53 -	54 -
55 1I-1, CHO-S p16 151009	56 1I-1, CHO-S p16 151009	57 1I-1, CHO-S p16 151009	58 5I-1, CHO-S p16 151009	59 5I-1, CHO-S p16 151009	60 5I-1, CHO-S p16 151009	61 6I-1, CHO-S p16 151009	62 6I-1, CHO-S p16 151009	63 6I-1, CHO-S p16 151009
64 1I-2, CHO-S p16 151009	65 1I-2, CHO-S p16 151009	66 1I-2, CHO-S p16 151009	67 5I-2, CHO-S p16 151009	68 1I-2, CHO-S p16 151009	69 1I-2, CHO-S p16 151009	70 6I-3, CHO-S p16 151009	71 6I-3, CHO-S p16 151009	72 6I-3, CHO-S p16 151009
73 1I-4, CHO-S p16 151009	74 1I-4, CHO-S p16 151009	75 1I-4, CHO-S p16 151009	76 5I-3, CHO-S p16 151009	77 5I-3, CHO-S p16 151009	78 5I-3, CHO-S p16 151009	79 6I-4, CHO-S p16 151009	80 6I-4, CHO-S p16 151009	81 6I-4, CHO-S p16 151009

Appendix II: Protocols

2.1.1 Leahy Lab Standard Purification Protocol – Neuregulin

Developed by Leahy Lab, typed by Lily Raines
Approximate Time to Completion: 5-6 days

Trx-His-Tev-Nrg ~ 17kDa
Cut Nrg ~ 6kDa

1. Transform Trx-His-Tev-NRG1β plasmid into Origami *E. coli* strain – positive clones will be resistant to ampicillin
- - -
2. Morning: pick colony, grow 2.5mL seed culture during the day
3. Use 0.2mL of culture to inoculate 100mL TB (terrific broth) + Amp, grow O/N shaking at 37ºC.
- - -
4. Add 20mL culture to 1L of TB + Amp (in 2L flask), grow at 30 ºC until OD_{600} = 1.5
Note: this will take 2 – 4 hours
4. When OD_{600} = 1.5, induce expression of EGF with 1mM IPTG (from 0.5M IPTG stock, or 240mg/1mL per L growth). Let grow O/N at 25 ºC.
5. Harvest cells by centrifugation at 5,000g x 10min – freeze at -80 ºC or lyse right away.
Note: take aliquot of induced cells to check for expression
- - -
6. Resuspend cell pellet in 20mL resuspension buffer.
Resuspension buffer: 50mM Na/KPO4 (pH 8.0), 500mM NaCl, 10mM imidazole
7. Lyse cells in French pressure cell, add 2uL of benzonase/DNAse 1 before lysis
Note: Max volume French press: 30mL.
8. Centrifuge at 14,000 rpm x 45 min at 4ºC
- - -
9. Equilibrate 2mL nickel resin with resuspension buffer
10. Apply cell lysate to equilibrated resin, batch bind with constant inversion at 4ºC for 1 hour.
11. Wash column with 5CV (10mL) of resuspension buffer
12. Elute protein with 3 – 4CV (6 – 8mL) elution buffer, collecting 1CV (2mL) fractions, using:
50mM Na/KPO4 (pH 8.0), 500mM NaCl, 50mM imidazole
13. Elute remaining protein with 3 – 4CV (6 – 8mL) elution buffer, collecting 1CV fractions, using:
50mM Na/KPO4 (pH 8.0), 500mM NaCl, 250mM imidazole
14. Confirm elution of NRG2β via Coomassie gel.
15. Pool fractions containing NRG2β, add EDTA to 0.5mM and TEV (50-500ug, depending on patience/stocks) and dialyze O/N at RT vs. 4L 20mM acetate pH 5.0.
- - -
16. There will probably be a lot of precipitate (mostly cut thioredoxin) in the dialysis bag, but most of the NRG2β should still be soluble. Spin at high speed to remove precipitate
Note: Work quickly! The precipitate will come back with time and the sample may need to be spun down again.

17. Assess if proteolysis is complete by SDS-PAGE.
18. Once complete, run sample over 6mL Resource S column. Elute over 15-20CV.
    Low salt buffer: 20mM acetate, pH 5.0
    High salt buffer: 20mM acetate, pH 5.0, 500mM NaCl

2.1.2 Leahy Lab Standard Purification Protocol – EGF

Developed by Kwangsoo Kim, typed by Lily Raines
Approximate Time to Completion: >1 week
Approximate Yield: 7mg EGF/2L cells

1. Transform pT7HT-EGF into BL21(DE3) *E. coli* strain – positive clones will be resistant to kanamycin

    Note: transformation efficiency may be low, easier to work from a glycerol stock

2. Let 5mL seed cultures grow O/N (grow 10mL seed cultures/1L cells final volume)
3. Add seed cultures to TB (terrific broth) + Kan, shaking at 37ºC (1L media/4L flask), and grow until OD_{600} = 1 (this will take >3hours)
4. When OD_{600} = 1, induce expression of EGF with 0.5mM IPTG (from 0.5M IPTG stock). Let grow for least 3 hours – O/N, then harvest cells by centrifugation at 5,000g x 10min.

- - -

5. Resuspend cell pellet (use 2x final volume pellet, ~ 30mL/2L cells) and stir at RT for 1 hour

    Resuspension buffer: 0.1M Tris pH8.0, 6M Guanidine-HCl

    Note: expect ~10g cell pellet/1L TB in 4L flasks

6. Lyse cells using sonicator – power setting 5, 20 mins total lysis time, 5 sec on/5sec off
7. Centrifuge at 15,000 – 20,000 rpm x 30 min at 4ºC

- - -

8. Equilibrate 15mL nickel resin with:

    0.1M sodium phosphate pH 6.0, 20mM imidazole, 0.5M NaCl, 8M Urea, 10% glycerol.

9. Apply spun cell lysate directly to nickel resin (do not batch bind/mix with resin)
10. Wash column with 3CV (45mL) of equilibration buffer
11. Elute protein with 5CV (75mL) elution buffer, collecting 1CV (15mL) fractions, using:

    0.1 M Tris (pH 8.0), 0.5 M NaCl, 0.25 M Imidazole, 8 M Urea

12. Confirm elution of EGF via Coomassie gel. Most protein will elute in 1st and 2nd fractions.
13. Pool fractions containing EGF and concentrate to 10mL using 3kDa spin concentrator

- - -

14. To reduce EGF, add 5mM DTT (from 500mM DTT stock) to sample and incubate at 37ºC for 30min-1hour (without mixing)
15. To refold protein, add EGF sample dropwise to refolding buffer to 10x volume (10mL sample to 90mL buffer) at RT

    Refolding buffer: 0.1 M Tris pH 8.3, 0.5 M NaCl

    Note: This can be done easily by adding the EGF sample to a gravity column and letting it drain, drop by drop, into the refolding buffer.

16. Dialyze the EGF O/N at 4°C in a buffer of 20 mM Tris (pH 8.0), 0.5 M NaCl, and 5% Glycerol

    Note: some protein will precipitate. After dialysis is complete, centrifuge sample at max speed to remove precipitate before proceeding to step 17.

- - -

17. Run a second nickel column, using 5mL pre-packed column on AKTA (as another concentration step). Wash with 5CV equilibration buffer, elute with 10CV elution buffer, collect 5mL fractions.

Note: these buffers ARE NOT the same as the first nickel column

Ni-2 Equilibration buffer: 20 mM Tris (pH 8.0), 0.5 M NaCl, 5% Glycerol

Ni-2 Elution buffer: 20 mM Tris (pH 8.5), 20 mM NaCl, 0.25 M Imidazole, 5% Glycerol

18. Determine which fractions contain EGF via Coomassie gel. Pool fractions containing EGF

19. Remove affinity tag by TEV proteolysis O/N at RT.

Use ~ protein:TEV (w/w) 50:1, or about 1mL Leahy Lab His-TEV

- - -

20. Monitor cleavage by Coomassie. Continue until >50% of protein is cleaved.

21. Dialyze sample O/N at 4ºC against: 20 mM Tris (pH 8.0), 0.5 M NaCl, 5% Glycerol

- - -

22. To remove His-TEV and to isolate cleaved EGF, run another nickel column. Use a pre-packed column and equilibrate with Ni-2 equilibration buffer [20 mM Tris (pH 8.0), 0.5 M NaCl, 5% Glycerol]. Load sample via syringe and collect flow-through

23. Concentrate flow-through to 5mL using spin filters, pore size 3kDa

- - -

24. Run concentrated sample over Superdex 75 16/60 column, equilibrated in:

20 mM Hepes, pH 7.4, 1 mM EDTA, 150 mM NaCl

25. Store at 4ºC as 40% glycerol solution (6mL EGF + 4mL glycerol), or at -80ºC long term

Desired final concentration EGF: ~ 1mg/mL

2.1.3 Leahy Lab Standard Purification Protocol – TEV/PreScission

Approximate Time to Completion: 2 days
Approximate Yield: >10mg

Notes:
1. Both proteins are not very stable in low salt. Prescission is more active in high salt.
2. Expression at 25 ºC is critical. Higher temperatures give insoluble protein and lower temperatures give less protein.
3. Ensure that protein isn't lost during the Ni2+ wash steps.
4. Prescission and can undergo at least 4 cycles of freeze-thaw without losing activity.

Purification
1. Grow the BL21(DE3)CodonPlus-RIL strain with the TEV-S219V plasmid or the Rosetta2 strain with pBrian-Prescsission plasmid in 2 L TB+Amp+Chloramphenicol at 37 ºC until OD600 ~1.5.
2. Induce with 1 mM IPTG and let the induction last overnight at 25 ºC.
- - -
3. Pellet the cells, then resuspend in
    35 mM PO43- pH 8.0, 400 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol,
    1uLDNase and 1uL Benzonase/10mL lysate.
4. Lyse via French Press.
5. Centrifuge at 20,000 x g for 50 min at 4 ºC. Filter the supernatant through 0.45 µm filter
6. Incubate the lysate with 12-15 ml Ni2+-NTA resin for 15 min at 4 ºC.
6. Pour the suspension into an empty column and drain.
7. Wash out unbound protein with 2 CVs of Ni2+ Bind Buffer.
8. Wash the column with 4 CVs
    20 mM PO43- pH 8.0, 40 mM NaCl, 25 mM imidazole, 10% glycerol.
9. Elute the column with 3 CVs of
    10 mM PO43- pH 7.0, 40 mM NaCl, 250 mM imidazole, 10% glycerol.
10a. For TEV: pool fractions and add 100% glycerol to 50% v/v final concentration at ~1 mg/ml TEV and freeze in 1 ml aliquots at -80 ºC.
10b. For Prescission: pool fractions and either freeze directly or dilute to 1 mg/ml with 20 mM PO43- pH 8.0, 400 mM NaCl, 10% glycerol and freeze at -80 ºC.
- - -

OPTIONAL: *S-column after Ni-NTA, better to skip this step!!! 95% pure after Ni-NTA and will take hit in final yield. If desired, run as below*
10. Dilute the elution 2 fold with 10 mM PO43- pH 7.0, 10% glycerol and load onto 6 ml Resource S column.
    Buffer A= 20 mM PO43- pH 7.0, 20 mM NaCl, 10% glycerol.
    Buffer B= 20 mM PO43- pH 7.0, 1 M NaCl, 10% glycerol
11. Elute with a gradient to 50% Buffer B.

2.1.4 Leahy Lab Standard Purification Protocol – EndoF

Developed by Samuel Bouyain
Approximate Time to Completion: 3-4 days
Approximate Yield: 50 - 100mg

Notes:
1. Endoglycosidase F1 has been cloned into pT7 with His6 and TEV site. Two versions of the protein have been made: one wild type and one with CysSer mutation (there is only one cysteine in the mature protein). The wild type and mutant endoglycosidase F1 have been named EndoF1 and,EndoF6, respectively. The following protocol was used to produce both enzymes and the protein will be referred to as EndoF throughout.
2. The tag could be removed by TEV but is left on so that it can be removed by Ni affinity chromatography after the deglycosylation reaction is over. The protein is not pure but could in theory be purified by ion exchange on Resource S at pH 7.0 (pl of 9.0). However, it seems unstable at low salt and will partly crash out in dialysis bag in 20 mM NaPi 7.0. Adding salt to 500 mM solves this problem.
3. EndoF cloned from *Flavobacterium meningosepticum*
4. Absorbance for 1 mg/ml His-TEV-EndoF: 0.856

Purification
1. Transform 1 ul of EndoF plasmid into BL21(DE3) cells. Add 50 ul of 50% glucose on the agar when plating the cells.
- - -
2. Take a swab of colonies on the plate and add to a 100 ml flask of LB-Kan (in a 500 ml flask), grow at. 37°C for 2 to 3 hours, shaking 225 rpm.
3. Inoculate 100 ml of culture to a 1000 ml flask of TB-Kan (in a 2Lflask), grow at 37°C until OD_{550} is about 2.0 (more is probably *fine).*
   This takes about 2 to 4 hours. Leaving the flasks in the incubator, decrease temperature to 30°C and grow for a further 1 hour.
4. Add IPTG freshly dissolved in water to a final concentration of 1 mM (e.g. dissolve 240 mg in 1 ml of water and add the whole solution to a 1000 ml culture). Grow overnight (16 to 20 hours).
- - -
5. Spin cells down. At this stage, they can be frozen at -80°C or lysed straightaway. Take an aliquot of induced cells to check for expression on a polyacrylamide gel.
6. Resuspend cells in 20 ml of
   500 mM NaCl, 10 mM imidazole, 20 mM Na/K P04 pH 8.0 (this is the loading buffer).
7. Add 200 units benzonase.
8. Lyse in French press with two passes.
9. Spin down lysate. 14,000 rpm for 45 minutes in SA-600 works.
10. Filter supernatant with 0.45 µm steriflip.
11. Add supernatant to 5 ml-column of chelating sepharose fast flow (Amersham) loaded with nickel sulfate
12. Wash with 5 CV of loading buffer.

13. Perform three elutions:
    3 CV of loading buffer with 50 mM imidazole,
    3 CV loading buffer with 100 mM imidazole
    3 CV loading buffer with 250 mM imidazole.
14. At this stage, run a gel. Pool 100 mM and 250 mM imidazole fractions.
15. Concentrate protein to less than 10 ml, some cloudiness may appear.
16. Filter protein using 0.45 μm filter and load onto Superdex 75 26/60 in
    20 mM NaPi 7.0, 500 mM NaCl.
17. Pool pure fractions (elution volume between 160 and 180 ml.)
18. Dilute to 1 mg/ml in reaction buffer with 50% glycerol, freeze at -80^{o}C

2.1.5 Leahy Lab Standard Purification Protocol – EndoH

Developed by Samuel Bouyain
Approximate Time to Completion: 2-3 days
Approximate Yield: 57mg/2L

Notes:
1. Endoglycosidase H has been cloned into pPROHEX HT (Amp resistance). The plasmid must transformed into Rosetta2 cells (Novagen) for efficient expression. These cells must be grown in the presence of chloramphenicol.
2. EndoH is cloned from *Streptomyces picatus*
3. MW Endo H is 29,000 Da

Purification
1. Transform 1 µI of EndoH plasmid into Rosetta2(DE3) cells (Novagen).
- - -
2. Prepare 4 culture tubes with 5 ml of LB with Amp and Chi (100 µg/ml and 34 µg/ml, respectively), inoculate each tube with a colony from the plate. Alternatively, one can start from frozen stock.
3. Grow at 37°C overnight, shaking 225 rpm.
- - -
4. Inoculate 1O ml of culture to a 1000 ml flask of TB-Amp/Chi (in a 2 I flask), grow at 37°C until OD_{600} is about 2.0.
5. Leaving the flasks in the incubator, decrease temperature to 25°C and grow for a further 1 hour.
6. Add IPTG freshly dissolved in water to a final concentration of 1 mM (e.g. dissolve 240 mg in 1 ml of water and add the whole solution to a 1000 ml culture).
7. Grow overnight (16 to 20 hours).
- - -
8. Spin cells down. At this stage, they can be frozen at -80°C or lysed straightaway. Take an aliquot of induced cells to check for expression on a polyacrylamide gel.
9. Resuspend cells in
    50 ml of 500 mM NaCl, 50 mM Na/K P04 pH 8.0 (this is the loading buffer).
10. Lyse in microfluidizer, rinse cell with 50 ml of loading buffer.
11. Spin down lysate. 16,000 rpm for 30 minutes in SS-34 works.
12. Filter supernatant with 0.45 µm steriflip.
13. Add supernatant to 8 ml-column of 1:1 slurry of His-select resin (Sigma) in loading buffer. Batch-bind for 1h on rotisserie.
14. Load lysate and resin onto column.
15. Wash with 5 CV of loading buffer with 20 mM imidazole.
16. Elute with 2.5 CV of:
    10 mM Tris pH 8.0, 40 mM NaCl, 10% glycerol and 200 mM imidazole.
    Keep an aliquot of the eluate for the gel.
17. Add eluate slowly to 40 ml of 20 mM Tris pH 8.0, 10% glycerol while stirring. Filter through 0.45 µm filter.

18. Load onto HiPrep Q XL column on akta equilibrated in 20 mM Tris pH 8.0, 10% glycerol.
19. Wash column with 3 CV of low salt buffer.
20. Elute with salt gradient to 500 mM over 12 CV.
21. Pool fractions.

    Check for aggregation by running an aliquot onto analytical gel filtration column. The last time the protein was produced, yield was 57 mg for 2 liters of culture with all the protein being monomeric (Mw - 29,000 Da).
22. Add glycerol to 50% and freeze at -80°C.

2.1.6 Leahy Lab Standard Purification Protocol – PNGaseF

Developed by Samuel Bouyain
Approximate Time to Completion: 2-3 days
Approximate Yield: 4mg/2L

Notes:
1. PNGaseF = peptide-N glycosidase F
2. PNGaseF has been cloned into pMal-p2x (maltose-binding protein with periplasmic expression, Amp resistance).
3. MBP-PNGaseF: 701 amino acids, M=77183.41 Da, isoelectric point 5.49,
4. Absorption of a 1 mg/ml solution at 280 nm in a 1 cm cuvette: 1.767.

Purification

1. Transform 1 µI of PNGaseF plasmid into BL21(DE3) cells (Novagen).
- - -
2. Prepare 4 culture tubes with 5 ml of LB with Amp (100 µg/ml), inoculate each tube with a colony from the plate. Alternatively, one can start from frozen stock. Grow at 37°C overnight, shaking 225 rpm.
- - -
3. Inoculate 10 ml of culture to a 1000 ml flask of TB-Amp (in a 2L flask) containing 2 g of glucose/L
4. Grow at 30°C until OD_{600} is about 1.5.
5. Leaving the flasks in the incubator, decrease temperature to 23°C and grow for 1 hour.
6. Add IPTG freshly dissolved in water to a final concentration of 1 mM (e.g. dissolve 240 mg in 1 ml of water and add the whole solution to a 1000 ml culture). Grow overnight (16 to 20 hours).
- - -
7. Spin cells down. At this stage, they can be frozen at -80°C or lysed straightaway. Take an aliquot of induced cells to check for expression on a polyacrylamide gel.
8. Resuspend cells in
50 ml of 250 mM NaCl, 25 mM NaP04 pH 8.0 and 0.5 mM EDTA (this is the loading buffer).
9. Lyse in microfluidizer, rinse cell with 50 ml of loading buffer.
10. Spin down lysate. 16,000 rpm for 30 minutes in SS-34 works.
11. Filter supernatant with 0.45 µm steriflip.
12. Add supernatant to 20 ml of amylase resin (New England Biolabs) as a 1:1 slurry in loading buffer (so 10 ml bed volume). Batch-bind for 1h.
13. Load lysate and resin onto column.
14. Wash with 10 CV of loading buffer.
15. Elute with 3 CV of loading buffer with 10mM maltose.
16. Desalt or dialyze eluate into 20 mM Tris pH 8.0.
17. Filter through 0.45 µm filter.
18. Load onto HiPrep Q XL or Resource Q column on akta equilibrated in 20 mM Tris pH 8.0.

19. Wash column with 3 CV of low salt buffer.
20. Elute with salt gradient to 1000 mM over 15 CV.
21. Pool fractions.

    Check for aggregation by running an aliquot onto analytical gel filtration column. The last time the protein was produced, yield was 4 mg for 2 liters of culture. The enzyme was active. One might be able to increase the yield by raising the temperature of the induction maybe to 25°C or 30°C, but there is a chance that this would overload the secretion system of E. coli, resulting in no active protein at all: it has to be tested.

2.1.7 Leahy Lab Standard Purification Protocol – tEGFR

Developed by Kwangsoo Kim, typed by Lily Raines
Approximate Time to Completion: 4 days
Approximate Yield: 100 – 200ug tEGFR/1L of cells

Note: store buffers at 4°C and top off DTT over time

Materials Required
1g 528-coupled CnBr beads per liter of cell homogenate
1.5mg (at least!) His-tag removed EGF
Multiple 0.45μM steriflips or larger vessel vacuum filters (for clarifying cell homogenate)
30uL total, 10uL Halt protease inhibitor cocktail/1mL lysate
tEGFR lysis buffer: 40mM Hepes 7.4, 1% TX-100, 20% glycerol
1L – 40mL 1M Hepes, 10mL TX-100, 200mL glycerol
Add Halt protease inhibitor cocktail (Pierce, 100 ul in each tube), 10 ul per 1mL lysate, before lysis.
Receptor buffer: 20mM Hepes 7.4, 150mM NaCl, 1mM EDTA, 0.5mM DTT, 0.03% DDM, 10% glycerol
1L – 20mL 1M Hepes stock, 30mL 5M NaCl stock, 2mL 0.5M EDTA, 1mL 500mM DTT, 0.3g DDM, 100mL glycerol
Wash solutions:
50mL receptor buffer + 1M NaCl = 10mL 5M NaCl stock to receptor buffer.
25mL receptor buffer + 1M Urea = 1.5g urea to receptor buffer.
tEGFR Superose 6 buffer: 20mM Hepes 7.4, 150mM NaCl, 1mM EDTA, 0.5mM DTT, 0.03% DDM, 5% glycerol, 1mM PMSF
1L – 20mL 1M Hepes stock, 30mL 5M NaCl stock, 2mL 0.5M EDTA, 1mL 500mM DTT, 0.3g DDM, 50mL glycerol, 10mL 100mM PMSF (made in isopropanol)

Purification
1. Thaw the cell pellets, add an approximately equal amount of lysis buffer to pellets (but at least 30mL!)
2. Lyse cells using a sonicator for 3 x 20s bursts at setting 3 (30-50% output) with 1 min off between sonications. Lyse with sample on ice in cold room.
3. Centrifuge cell homogenate at 14,000 x rcf for 30 min at 4°C
   Note: Can leave lysate to rotate in the cold room for 1 hour for more extraction time?
4. Filter supernatant using vacuum driven filters (steriflips or large ones) with 0.45μM cut-off. (0.22μM will work but will take longer).
   Note: Approximate time: 45 minutes – 1 hour with large volume (500mL) filters.
5. Apply filtered supernatant to 528-coupled beads, inverting overnight at 4°C.

- - -

6. Transfer beads to a vacant gravity/econocolumn.
7. Wash the beads as follows:

- 25mL receptor buffer
- 25mL receptor buffer + 1M NaCl
- 25mL receptor buffer
- 25mL receptor buffer + 1M NaCl
- 25mL receptor buffer
- 25mL receptor buffer + 1M Urea
- 50mL receptor buffer

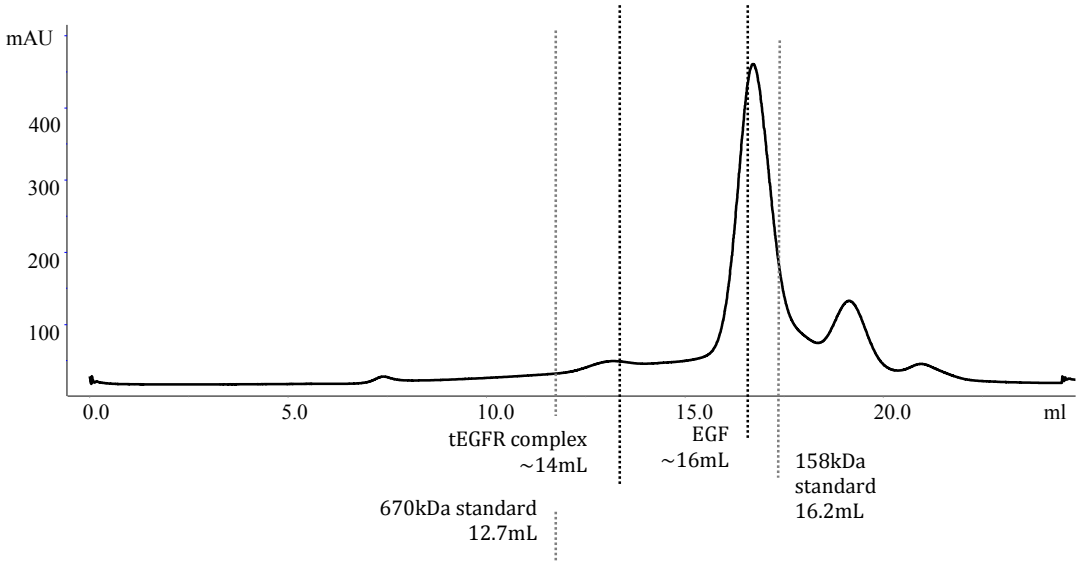
Approximate total time (FT to first elution) = 3 hours

8. Transfer beads to a 15mL Falcon tube, spin down at 1,200 x rcf for 5 minutes at 4ºC, and remove leftover wash solution.
9. To elute tEGFR, add an equal volume of receptor buffer with EGF at 50µg/mL, then incubate with inversion at **room temperature** for 30 minutes.
   For 1L beads, expect to add 2 – 3.5mL at 50µg/mL for 100µg EGF per elution.
10. After 30 minutes, spin solution at 1,200 x rcf for 5 minutes at 4ºC and collect first ‘elution fraction.
11. Repeat for a total of twelve elutions. Approximate time: 7 hours
    Expect ~50% capture on 528 beads
12. Run an SDS-PAGE gel to determine which elutions contain tEGFR
13. Pool all tEGFR containing solutions.
14. To deglycosylate, add 10µL of EndoH and EndoF per 10mL protein and let incubate overnight with inversion at 4ºC. Minimum incubation time is 2 – 3 hours.

- - -

15. Concentrate deglycosylated tEGFR to <u>240µL</u> to load onto Superose 6 analytical gel filtration column.
16. Run Superose 6 column at 0.7mL/min and collect 0.5mL fractions. Determine which fractions contain tEGFR by SDS-PAGE.

*** NOTE *** 2 tEGFR: 2 EGF dimers Ve ~14mL (20mAU?) and Ve for free EGF ~16mL



2.2.1 Leahy Lab Technique Protocol – ELISA Plate Preparation

Developed by Patti Longo and Min-Sung Kim, typed by Lily Raines
Approximate Time to Completion: 3 hours
Makes 30 plates

Materials Required
Corning #9018 EIA/RIA 96-well Flat bottom plates
Corning Plate Sealers, #3095
~5mL Dialyzed calf serum (DCS) of fetal calf serum (FCS)
2.25mg HGH-B monoclonal antibody (protein A purified from mouse ascites)
2L Phosphate buffered saline 0.1% Tween-20 (PBST) – use from carboy near Lily's bench
1L 50mM Sodium Carbonate Buffer, pH 10.4
- 4.24g Na_2CO_3 + 0.84g $NaHCO_3$ per 1L H_2O. Set pH to 10.4
300mL mAb solution for coating plates (need ~10mL mAb solution per plate)
- 7.5ug/mL HGH-B mAb in 50mM sodium carbonate buffer
300mL plate blocking solution (need ~10mL blocking solution per plate)
- 1% DCS or FBS added to 50mM sodium carbonate buffer; 3mL in 300mL total volume

Preparing ELISA plates
1. Coat plates by adding 100uL of mAb solution to each well of a 96-well plate
   - apply with multichannel pipette from plastic trough for fast application. Work quickly!
2. Seal and leave plates at 37ºC for 1 hour
   - stack plates for easier transport, use removable plastic plate seal for topmost plate during all incubations to prevent plates from drying out.
3. Flick coating solution out of plates into sink and blot each plate one time with paper towel.
   DO NOT wash with PBST
4. Add 100uL DCS/FCS blocking solution to each well
5. Cover and leave plates at 37ºC for 1 hour
6. Wash plates 5 times with PBST, using wash apparatus near Lily's desk, store at 4ºC before use

Notes
- To prevent plates from drying out over long-term storage, keep plates in sealed boxes with a moist paper towel inside and a plate seal on the topmost plate.
- Plates can be stored for at least two months – one year, *as long as the wells don't dry out.*

2.2.2 Leahy Lab Technique Protocol – ELISA

Developed by Patti Longo and Min-Sung Kim, typed by Lily Raines
Approximate Time to Completion: 3 hours
Expected volumes listed per ELISA plate.

Notes:
- For a sample with ~0.1mg/L expected hGH concentration, dilute by 10 fold to stay within hGH standards. If approximate levels unknown, try 1x, 10x, and 100x dilutions.
- All samples are done in duplicate.
- Antibody solutions and HBSS/1%FCS can be made ahead of time and are stored in Jackie's refrigerator at 4°C
- Dispense antibody, TMB, and sulfuric acid solutions from plastic troughs using multichannel pipettes for easy and quick sample application.

Materials Required
Corning #9018 EIA/RIA 96-well Flat bottom plates
Corning Plate Sealers, #3095
25mL HBSS/1%FCS or 1%DCS
- HBSS = Hanks balanced salt solution, FCS/DCS = dialyzed or fetal calf serum

30uL Leahy lab human growth hormone stock for standards
- Leahy lab hGH stock is 640ng/mL with aliquots stored at -80°C, can store at -20°C

10mL of 1:10,000 α-hGH polyclonal antibody diluted in PBST
- α-hGH pAb From Research Diagnostics (#RDI-HGHabrX1)

10mL of 1:10,000 Goat α-rabbit IgG conjugated horse radish peroxidase (GAR-HRP)
- GAR-HRP From Santa Cruz biotech, #SC-2301)

10mL 3,3',5,5' tetramethyl benzidine (TMB, Sigma #T-8665)
- put at RT after adding samples to plates.

5mL 1M Sulfuric acid
200mL Phosphate buffered saline 0.1% Tween-20 (PBST) – use from carboy near Lily's bench

ELISA Assay
1. Serially dilute hGH standards in HBSS/1%FCS for 200uL of standards at 32, 16, 8, 4, 2, 1, 0.5, and 0ng/mL
   - If using Leahy lab stock at 640ng/mL, first dilute 10-fold into 300uL for 64ng/mL, Then add to 300uL of HBSS/1%FCS for the 32ng/mL sample. Mix thoroughly, then repeat until have 300uL of all hGH standards, taking 250uL of diluent for 0ng/mL sample.
2. Add 100uL (or desired volume) of hGH-containing samples diluted in HBSS/1%FCS to wells in a pre-coated 96-well plate.
3. Incubate, covered with a plate seal, at 37°C for 45 minutes. Then put TMB at RT
4. Wash plate 5 times with PBST. Flick out excess wash buffer.

5. Add 100uL of 1:10,000 dilution of polyclonal α-hGH to each well.
6. Seal and leave plates at 37°C for 30 minutes
7. Wash plate 5 times with PBST. Flick out excess wash buffer.
8. Add 100uL of 1:10,000 dilution of GAR-HRP to each well.
9. Seal and leave plates at 37°C for 30 minutes
10. Wash plate 5 times with PBST. Flick out excess wash buffer.
11. Add 100uL of TMB to each well and wait for standards to develop a blue color, ~3-5 minutes.
    - Want to see a hint of blue in 0.5 - 1ng/mL samples and none in 0ng/mL sample.
12. Stop reaction by adding 50uL of 1M sulfuric acid to each well.
13. Read plate at 450/655nm, with 5 seconds of mixing before detection.
14. Fit standards with a logistic fit, read-out from Leahy lab set-up will be in ug/L

– confirm in new space!

2.2.3 Leahy Lab Technique Protocol – Western Blot

Semi-Traditional Western
Total time: ~4 hours
Traditional Western
Total time: 2 days

1. Run acrylamide gel with desired protein samples, using Western compatible ladders
   MagicMark XP Western Standard, prestained: SeeBlue…
2. Transfer using iBlot system or traditionally
   iBlot: follow instructions by instrument
   Traditional: combine 50mL 10x transfer buffer, 125mL methanol, 325mL ddH2O.
   Run at 35V 120mA for 1.5 hours.
3. Block in 15-20mL of 5% BSA or 5% powdered milk in ELISA wash buffer
   at least 1 hour, up to overnight
4. Add primary antibody directly to BSA/milk solution, incubate with shaking for 30mins
   See "Leahy Lab Western Antibody Dilutions" document
5. Wash with ELISA wash buffer 5 minutes, discard buffer, then repeat for a total of 3x 5 minute washes (15 minutes total)
6. Add secondary antibody to 15-20 mL of 5%BSA or powdered milk, incubate blot for 30 mins
7. Wash as in Step 5
8. Dry blot between paper towels/chemwipes. Incubate in developing solution for 5 minutes
9. Analyze by film or Typhoon phosphorimager

Notes:
ELISA Wash buffer: PBS + 0.1% Tween 20.
Traditional 10x Transfer buffer:
14.5g Tris base, 72g glycine, ddH2O to 1L.

Developing with ECL-Plus and imaging on the Typhoon (filter 520BP40 laser457nm) provides semi-quantitative results.

2.2.4 Leahy Lab Technique Protocol – Standard Western Dilutions

Key
GAR: goat a. rabbit
GAM: goat a. mouse
(T): traditional western
(S): SnapID
* Both FLAG mAb's same clone, dif purification, both suitable for westerns.

Western vs....	Primary Antibody	Source and Cat No	Dilution	Secondary Antibody	Dilution
Commonly Used					
hGH	pAb (rabbit)	lab	(T) 1:10,000 - 1:20,000	GAR	(T) 1:10,000 - 1:20,000
Myc	9e10 (mouse)	SC-40	(T) 1:1000 - 1:5000	GAM-IgG1 HRP	(T) 1:2000
P-Tyr	4G10 (mouse)	Millipore 05-321	(T) 1:2000	GAM-IgG2b HRP	(T) 1:2000
HA	3f10 (rat)	Roche 1 867 423	(T) 1:1000	goat a. rat	(T) 1:3000
His6	(mouse)-HRP		(T) 1:2000	-	-
Strep Tag	HRP-Conj Streptactin	IBA 2-1502-001	(T) 1:1000 - 1:2000	-	-
SBP	HRP-Conj Streptactin	IBA 2-1502-001	(T) 1:10,000 to 1:20,000	-	-
FLAG*	M2 (mouse)	Sigma F1804 ($)	(T) 1:3000	GAM-IgG1 HRP	(T) 1:3000
FLAG*	M2 (mouse)	Sigma F3165 ($$)	(T) 1:3000	GAM-IgG1 HRP	(T) 1:3000
EGFR and related					
EGFR	3H2094 (mouse)	SC-71033	(T) 1:200 - 1:1000 (S) 1:1000 - 1:3000	GAM-IgG1 HRP	(T) 1:1000 (S) 1:3000
Her2	Neu(9G6) (mouse)	SC-08	(T) 1:3000 (S) 1:3000	GAM-IgG1 HRP	(T) 1:1000 (S) 1:3000
IGF1R and related					
IGF1R alpha	N-20 (rabbit)	SC-712	(T) 1:15,000	GAR	(T) 1:15,000

			(S) 1 to 300		(S) 1:3000
IGF1R beta	C-20 (rabbit)	SC-713	(T) 1:15,000 (S) 1 to 300	GAR	(T) 1:15,000 (S) 1:3000

2.2.5 Leahy Lab Technique Protocol – CNBr Bead Coupling

Developed by Kwangsoo Kim, adjusted by Zhihong Wang, typed by Lily Raines
Approximate Time to Completion: 4 hours, overnight incubation.
Approximate Amount of Beads/prep: 0.5g 528-coupled resin/1L HEK 293's
All steps written for coupling 1g of beads

Materials Required:

STORE ALL SOLUTIONS AT 4ºC BEFORE USE

- 1g CNBr-activated beads (GE healthcare), volume ~ 3.5mL/1g
- 528 EGFR antibody (stored in 0.1M NaHCO3 pH 8.0, 0.5M NaCl, at 5 – 10mg/mL)
  - 10mg antibody/1g beads
- 1 mM HCl - ~150mL/1g bead coupling
- Coupling buffer: 0.1 M NaHCO3 pH8.3, 0.5 M NaCl - ~150mL/1g beads
- Quenching buffer: 0.1 M Tris pH 8.0 – ~50mL/1g beads
  (1M ethanolamine pH 8.0 also works)
- Storage buffer (20 mM Hepes pH 7.4, 150 mM NaCl - ~100mL/1g beads
- Receptor buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 10% Glycerol, 0.5 mM DTT, 0.03% DDM – for tEGFR purification later. At LEAST 1L/prep

1. Weigh out desired amount of sepharose beads
2. Add 10 resin volumes of cold 1mM HCl
3. Incubate with constant inversion (i.e. on a tube rotator) for 30min at 4ºC
4. Divide the resin between two 15mL falcon tubes (~0.5g beads each), then add 10mL of 1mM HCl, invert a few times, then centrifuge at 1,200 x g for 3min at 4ºC
5. Repeat for a total of three washes.
6. Add 10mL of coupling buffer to each tube and wash similarly as in steps 4 and 5 for a total of three washes.
7. Re-check the volume of beads, then add an equal volume of coupling buffer
8. Add the 528 antibody solution to each tube (adding 5mg/0.5g beads) and incubate at room temperature with constant inversion for 2 – 4 hours.
9. Note final antibody concentration of the above reaction.
10. After 2 – 4 hours, wash the resin with 10mL of coupling buffer, then spin at 1,200xg for 3min at 4ºC and retain the supernatant. Check the A280 of the wash supernatant.
    If A280 < 0.1, proceed with wash and storage steps. Otherwise continue coupling.
11. Wash as in step 6 with 10mL of coupling buffer for a total of three washes
12. Block unbound active sites on beads by washing with at least 3 resin volumes of quenching buffer for a total of three washes.
13. Let beads invert overnight (or sit at room temperature for 2 hours) at 4ºC.
14. The next day, wash the coupled resin as in step 6 with 10mL of storage or receptor buffer for a total of three washes. Store at 4ºC until use.

2.2.6 Leahy Lab Technique Protocol – Antibody Digestion

Developed by Zhihong Wang, notes from Lily Raines
Protocol written specifically for digestion of cetuximab, should be broadly applicable.

Solutions:

200mM L-Cysteine in 75mM PO_4^{3-} , pH7.0, 75mM NaCl
500mM Iodoacetamide in 75mM PO_4^{3-} , pH7.0, 75mM NaCl
Papain stock: 25mg/ml

Protocol:

1. Digest 2mL Cetuximab (7.7mg/ml) with 1% papain (6uLof papain stock) in the presence of 10mM Cysteine at 37°C for 3hr
2. Stop the reaction by adding 50mM Iodoacetamide (200uL stock to 2mL reaction buffer), incubate at 37°C for 15-30 min, then put the sample on ice. Load 1uL reaction buffer to non-reducing SDS-PAGE gel to check how the reaction goes.
3. Dilute 2mL reaction buffer to 10 mL with 10mM NaCl, 20mM Tris pH8.0. Use 10mL superloop to load the proteins onto Mono Q™ 5/50 GL column. Check the fractions with Coomassie stain.

   Fab will not bind to the Q column and will be in the FT!

   Note: I loaded the sample three times to Mono Q column.
4. Combine all the fractions and concentrate with Mw 10,000 cut-off concentrator to 1 mL.

   Note: if you load a 5mL HiTrap Q column (freshly cleaned) and flow at 1mL/min you can get all the Fc to bind in one go.
5. To get more pure Fab, size-exclusion column can be used after Mono Q
   Note: Zhihong and Lily skip this step, but recommended in some protocols/some antibodies.
6. Concentrate Fab to a final concentration of ~6mg/ml, add 40% glycerol, aliquot (~100ul per aliquot), and keep them @-80.

Notes:

Since Fc and Fab have similar size, make sure you know capture the right protein. (Fc is slightly bigger than Fab on a 10% SDS-PAGGE non-reducing gel)

2.2.7 Leahy Lab Technique Protocol – BCA Assay

Abbreviated notes from manual
Approximate Time to Completion: 45 minutes

"Microplate method"
1. Prepare 0.2mL working reagent (WR) per sample (including standards)
   1 part B to 50 parts A
2. Prepare 8 standard dilutions, prepare 25uL

standard	*standard ug/mL*	*uL stock*	*uL diluent*	*Total volume*
A	*2000*	*25*	*0*	*25*
B	*1500*	*18.75*	*6.25*	*25*
C	*1000*	*12.5*	*12.5*	*25*
D	*750*	*12.5 B*	*12.5*	*25*
E	*500*	*12.5 C*	*12.5*	*25*
F	*250*	*12.5 E*	*12.5*	*25*
G	*125*	*12.5 F*	*12.5*	*25*
H	*25*	*5 G*	*20*	*25*
I	*0*	*0*	*25*	*25*

3. Take 10 - 25uL each unknown and each standard into separate labeled tubes
4. Add 200uL WR to each, keep at 37oC for 30mins
5. Read A_{562} on nanodrop, blank instrument with water and subtract "blank" standard from all standards and unknowns.

Note: Read all samples within 10 minutes, as BCA continues to develop

2.2.8 Leahy Lab Technique Protocol – Cell Surface Biotinylation

Developed by Jackie McCabe, modified from EZ-Link Sulfo-NHS-SS-Biotin Protocol

Materials

Biotin Solution – 1.4mL/well of a 6-well plate; 10mL/T75 flask

- Dilute 0.25mg EZ-link Sulfo-NHS-SS-biotin/mL of PBS

PBS on ice – 4mL/well
Quench solution: 50mM Tris pH 7.4 – 1mL/well
Lysis buffer: RIPA + 1mM Na_3VO_4 – 25uL/well
Streptactin Resin – 20uL/well

Protocol

1. Plate cells at $1x10^6$/well, wait at least two hours or overnight to proceed
2. Wash cells two times with ice-cold PBS
3. Bring to cold room
4. Add 1.4mL of biotin solution per well, leave at 4ºC for 15 minutes
5. While still in cold room, quench with 0.933mL of 50mM Tris pH 7.4
6. Cells will become non-adherent, so suspend cells and spin at 1,000 x g for 15 minutes
7. Wash with ice cold PBS, spin and repeat for a total of two ice cold PBS washes
8. Add 25uL lysis buffer/sample
9. Add each sample to 10 – 20uL of streptactin resin, mix for 2 hours at RT
10. Spin, then wash resin with 1% NP-40, 1% SDS in PBS
11. Spin, then wash resin with 0.1% NP-40, 0.5M NaCl in PBS
12. Boil beads for (30 seconds?), run a gel to determine if protein was successfully biotinylated.

Notes

- Dispose of streptactin resin after use
- MW of Sulfo-NHS-SS-Biotin: 607g/mol
- Source protocol recommends 80uL of 10mM Biotin (6mg/1mL) per milliliter of for $25x10^6$ cells/mL
  > for $2.5x10^6$cells/mL, use 0.6mg/1mL biotin; for $1x10^6$ cells/mL use 0.25mg/mL biotin
- Source protocol recommends 15mL biotin be quenched with 10mL quench solution
  >1.4mL biotin should be quenched with 0.933mL 50mM Tris pH 7.4

2.2.9 Leahy Lab Technique Protocol – Immunoprecipitation

Developed by Jennifer Kavran and Jackie McCabe
Approximate time to completion: ~ 8 hours

Materials

RIPA supplemented with (250uL per well + extra for BCA dilutions):

- 1uL benzonase/10mL RIPA
- 1mM PMSF; 100uL of 100mM PMSF stock/10mL RIPA
- 1mM Na3VO4; 14uL of 0.7M Na_3VO_4 stock/10mL RIPA
- 10mM iodoacetamide (if using to prevent additional cysteine cross-linking); 200uL of a 0.5M stock/10mL RIPA
    - Make 0.5M iodoacetamide stock solution right before use, not stable

Dynabead wash solution: RIPA + 1mM Na_3VO_4 – 3mL/sample
Ham's F12 + 1mg/mL BSA – 8mL/well
PBS – on ice, 2mL per well
Anti-HA conjugated Protein G dynabeads – 20uL/sample

- Add 200ng anti-HA to 0.6mg protein G dynabeads in PBS + 0.01% Tween 20
- rotate 30 mins at RT, store at 4°C

Dynabead magnets, etc.
2X SDS loading buffer + fresh 10% BME – 30uL/sample

- freshly added 10% β-mercaptoethanol: bottle <1yr old, kept at 4°C

Protocol

1. Plate cells at 1.0 x 10^6 cells/well, wait at least two hours to proceed
2. Wash cells three times with 2mL of Ham's F12 + 1mg/mL BSA
3. Serum starve in Ham's F12 + 1mg/mL BSA for 3 hours at 37°C
4. Stimulate with appropriate ligand for 5 minutes at 37°C
   IGF1 – 20nM | 200nM insulin | 100ng/mL EGF
5. Wash cells with ice-cold PBS, 2mL/well
6. Lyse cells with 250uL RIPA/well at 4°C for 30 minutes.
7. Clarify lysates by spinning at maximum spin at 4°C for 15 minutes
8. Determine protein concentration using BCA assay
   – see separate protocol
9. Normalize lysate volumes relative to most dilute sample, dilute others with RIPA + Na_3VO_4 to 200uL
10. Add 200uL of normalized lysate to 20uL of HA-conjugated protein G dynabeads.
11. Incubate O/N at 4°C **OR** 2 hours at RT
    This depends on how sensitive your protein is to degradation. Try O/N first and then 2 hours if yields are low
12. The next day wash three times with 1mL RIPA + 1mM Na_3VO_4
    For washes, let reactions stir for 5 minutes before pull-down
    For dynabeads, allow 2 minutes for beads to bind to magnet to reduce sample loss
    For protein G beads, spin at recommended speed and time (protein G manual)
13. Add 30uL of 2x SDS buffer + 10% fresh BME to elute

Notes:
- For dynabeads, always dispose of beads after use
- For protein G beads, spinning beads for 15 – 30 minutes at maximum speed after elution improves gels
- Jennifer always kept samples at 4ºC b/c IGF1R is super sensitive to proteolysis.
- For Kavran et al, JMK froze samples at –20 overnight after elution (where stimulation, lysis, pull-down for 2 hours, and elution were all performed on the same day) and ran gels the next night.

2.3.1 Leahy Lab Cloning Protocol – General Cloning Protocols

PCR Reactions
10uL Phusion HF buffer
5uL DMSO, if needed
2uL dNTP (10mM working stock)
1uL each template
2uL each primer (1:10 dilution of 100nM stock)
0.5uL enzyme
>ddH2O to 50uL

PCR Tanneal – Tm lower + 5
Agarose gels – 1g/100mL TAE buffer, ~6uL ethidium bromide

Cutting Vectors (for pre-cut stocks)
To 50µL, add
- 1uL each enzyme
- 6uL BSA, 100ug/mL (1x, NEB)
- 6uL NEB double digest compatible buffer

Leave reaction for 4 hrs at 37ºC for vectors, 1 hour for inserts
Add 1 uL CIP (calf intestinal phosphatase) if using for vector stocks (to prevent re-ligation)
Leave for 30 mins at 37ºC
Elute on agarose gel, extract, get concentration, then ligate

Ligations (insert into vector)
Use ~[cut vector] to [cut insert], 20uL reaction mix
- 1uL vector
- 2uL bfr T4 ligase
- 0.5uL T4 ligase
- __uL insert
- ddH2O to 20uL

Leave at 16ºC O/N or at RT 1 hour
Most transformations: half of ligation mixture (10uL) to 100uL DH10B

Enzyme Digestions (confirm proper insert size)
20ul/well
- 2uL compatible digest buffer
- 2uL BSA
- 15uL ddH2O
- ~1uL template/well
- Negligible enz (1uL each to overall mix)

Make Bfr/BSA/H2O mixture for all wells, add 1uL each enzyme to master mix.
Incubate at 37ºC for 2 hours
Run agarose gel.

Primer design
60bp> for IDT, try and end with one or two G/C at 3' end, no more than two. ~20-30 bp fine sqn
If adding restriction site, give 6-8 bp random DNA after 5' end. A/T better, don't change Tm much.

5x LDS recipe	**For 25mL 5x LDS**
5% LDS (lithium dodecyl sulfate)	1.25g LDS
0.1% bromophenol blue	0.025g bromophenol blue
200mM Tris pH 6.8	5mL 1M Tris-HCL
1.25M sucrose	10.69g sucrose

For 25mL: pH a little water and Tris first! Turns yellow/orange if too acidic, green if not enough liquid

2.3.2 Leahy Lab Cloning Protocol – Bailey Lab Multi-site Mutagenesis

1. Assemble mixture in the given order

	[Final]	Control
dH2O	to 25 uLs	to 25 uLs
10x Taq Ligase Buffer	1x	1x
ATP (@ pH ~7.0)	2 mM	-
dNTPs	1 mM each	-
Gel purified primers (1-5)	0.2 uM each	-
Template DNA	~ 100 ng	~ 100 ng
Pfu Ultra DNA polymerase	1 uL	-
NEB Taq ligase	1 uL	-
NEB T4 Polynucleotide kinase	1 uL	-
Total	**25 uL**	**25 uL**

2. Add 1 uL of NEB DpnI directly to the mixture and incubate the reaction for 4-6 hours @ 37C (O/N if convenient)
3. Transform 5 uL of the digested DNA into E. coli
4. Next day-Grow 2-3 colonies and test digest/colony PCR to make sure that your entire gene is intact. Because this protocol uses primers that anneal on a single (same) template strand, I have found that in case of some of the colonies, the plasmids are truncated (and usually your gene is truncated). So I make sure the digestion products from my test digests look identical to results I expect in case of the template DNA.
5. If my test digestions make sense, I send 2 samples for sequencing.
6. When I use 5 primers (for 5 mutations), I usually find 4-5 of the sites mutated (different combinations of sites mutated in DNA from different colonies).

Bailey Lab observations

1. I get better results (higher number of colonies in my mutagenesis plate compared to my control plate) when I gel purify each of the primers (Denaturing UREA gel)
2. I get more colonies when I do the mutagenesis with shorter plasmids (overall length of the plasmid does have an effect)

2.3.3 Leahy Lab Cloning Protocol – Around-the-Horn Cloning

Protocol from Tim Blower, Berger Lab.
Typed by Lily Raines, updated by Jennifer Kavran
For reference: http://openwetware.org/wiki/%27Round-the-horn_site-directed_mutagenesis

General Notes:
Each primer should be ~18bp OF COMPLIMENTARY SEQUENCE**
DON'T use a Taq polymerase, Pfu Ultra works well.
THE MORE DNA GOING INTO THE LIGATION THE BETTER, so if no colonies after first round try running multiple of PCR rxn and purifying into 16uL ***

1. Primer Phosphorylation

Perform the following reaction separately for each primer.

Reaction Mixture:
5uL PNK (T4 polynucleotide kinase, NEB) buffer
0.5uL 100uM MgSO4
2.5uL primer at **200uM** (final concentration = 10uM)
1uL ATP at 200mM, pH 7.5
1uL PNK
dH_2O to 50uL final volume

Incubate reaction at 37 °C for 1 hour, heat kill the PNK at 95 °C for 5 minutes.

2. Site-directed Mutagenesis

Using pretreated primer mixture, set up the following reaction

Reaction mixture:	PCR Program
5uL 10x reaction buffer	1. 95°C – 1 minute
2.5uL pretreated primer 1 reaction (at 10uM)	2. 95 °C – 30 seconds
2.5uL pretreated primer 2 reaction (at 10uM)	3. 55 °C – 30 seconds
5uL dNTPs (at 2 - 5mM)	4. 68 °C – (30 sec/kb)
2uL template DNA	5. Repeat steps 2 – 4 x 25
1uL Ultra II Pfu	6. 68 °C – 5 minutes
dH_2O to 50uL final volume	12 °C – hold

** PFU ULTRA II SAYS 15s/KB BUT TIM THINKS 30S/KB WORKS BETTER
After PCR is complete, add 1.5uL DpnI and incubate at 37 °C for 2 hours
Run products on an agarose gel, gel extract band in ~ 16uL

3. Ligation

Ligate (using T4 DNA ligase as written elsewhere) either A) at RT for a few hours or B) overnight at 16 °C.
Use whole ligation mixture to transform in competent cells.

2.3.4 Leahy Lab Cloning Protocol – Using Bacmid for Insect Cell Transfection

Developed by Matthew Ward and Jennifer Kavran, typed by Lily Raines
Modified from Invitrogen Bac-to-Bac manual (Version E, Jan 19 2009)

Bacmid Transformation
Use Kanamycin (50ug/mL) + Gentamicin (7ug/mL) + Tetracycline (10ug/mL) plates.
Before use, add:
50uL of 40mg/mL XGal in DMSO
5uL of 40mg/mL IPTG in water
1. Add 1uL plasmid to 90uL DH10Bac
2. Incubate 30 mins on ice
3. Heat shock at 42 ºC for 30 seconds
4. 2 minutes on ice, then add 1mL LB and outgrow at 37 ºC for 4 hours (vs. typical 1 hour)
5. Spin cells down, resuspend in LB and plate ~200uL
6. Grow at 37 ºC for two days
7. Pick white colonies.

For bacmid purification, grow white colonies in 5mL of Kan + Gen + Tet at 37 ºC O/N
Notes: Kan stock – 1000x; Gentamicin stock stored at RT – 10mg/mL; Tet stock -20 covered – 4mg/mL
> Use 5uL Kan stock; 3.5uL Gentamicin stock; 12.5uL Tet stock

Bacmid Purification
Supplies Needed
100% isopropanol – 700uL /rxn
70% ethanol – 700uL + /rxn
TE buffer (miniprep kits) – 50-100 uL/rxn
Standard miniprep kit buffers, all standard volumes, or:
Resuspension Buffer – 15mM Tris-Cl pH 8.0, 10mM EDTA, 100ug/mL RNaseA
Lysis Buffer – 0.2M NaOH, 1% (w/v) SDS
Precipitation Buffer – 3M KCH3COO (potassium acetate) pH 5.5

1. Pick a **white** colony, grow O/N in 5mL of Kan + Gen + Tet at 37 ºC
2. Spin down cells, 5000 rpm for 5 mins, decant media and keep cell pellet
3. Resuspend in 250uL resuspension buffer
4. Add 250uL lysis buffer, mix by swirling, incubate at RT for 5 mins
5. Add 250uL precipitation buffer, mix by swirling
6. Transfer rxn to 1.5mL Eppendorf tube
7. Spin 14000 rpm x 10 mins at 4 ºC
8. Transfer supernatant to FRESH 1.5mL tube, discard white pellet (avoid transferring precipitate)
9. Add 700uL isopropanol to each tube, mix by inverting gently, incubate on ice 5 minutes

10. Spin 14000 rpm x 10 mins at 4 ^{o}C
11. Decant supernatant gently, retain pellet
12. Wash with 700uL 70% ethanol
13. Spin 14000 rpm x 10 mins at 4 ^{o}C
14. Decant sup gently. **Multiple ethanol washes are encouraged but not necessary**
15. Air dry pellet in tissue culture hood for at least 20 minutes
16. Resuspend pellet in 50 – 100uL TE buffer

Bacmid Transfections into Sf9 Cells

1. Plate Sf9 cells at 0.8 x 10^{6} cells/mL
(wait at least an hour to proceed)
2. Change media in plates to plating media
[1.5mL supplemented Grace's media + 10% FBS] + [8.5mL unsupplemented/basal Grace's media]
Note: Graces un/supplemented media and plating media should be prepared and stored in 50mL aliquots. Need 12mL of media per plate.
3. For each well to be transfected:
Add 2uL of 10x DNA to 100uL of Grace **un**supplemented media.
Add 8uL of Cellfectin II to 100uL of Grace **un**supplemented media.
4. Mix the DNA and Cellfection solutions together, set for 30 minutes at RT
5. Add mixture to cells
6. After 4 hours, change media to ISFM (insect serum free complete media)
Can be Leahy Lab ISFM or Sf93, insectagrow, Grace sup + 10% FBS
7. After 3 days, look for infection

Baculovirus Amplification and Expression in Sf9

1. Produce P1
Day 1 – transfect as above
Day 2 – x
Day 3 – Harvest P1 of virus
Decant media, add 2% FBS to store media + virus. Dispose of plated Sf9 cells
2. Produce P2
Day 1 – Add 0.5mL P1 to 25mL of Sf9 cells at 1.5x10^{6} cells/mL in a T-75 flask
Day 2 – x
Day 3 – Harvest P2 of virus
Decant media, add 2% FBS to store media + virus. Dispose of Sf9 cells
*Note: assume titer of **3x10^{6}** gives an MOI (multiplicity of infection) of 0.1*
2. Produce P3
Day 1 – Add 5mL P2 to 250mL of Sf9 cells at 2.2x10^{6} cells/mL in small spinner flask
Day 2 – x
Day 3 – Harvest P3 of virus
Decant media, add 2% FBS to store media + virus. Dispose of Sf9 cells
*Note: assume titer of **5.5x10^{6}** gives an MOI of 0.1*

> Innoculum required, in mL = [MOI x (number of cells)]/titer – we don't do the plaque assay

Titer ~=$1x10^6$ – $1x10^7$ – $1x10^8$

MOI: use 0.05 – 0.1 for virus; 1 – 5 for protein expression. Test 0.5, 1, 2, 10

Maintain Sf9 cells

- Grow in Leahy Lab ISFM + PenStrep (dilute as directed, not at half strength per manual)
- Split cells at ~$2x10^6$/mL, split back to 0.1 – $0.5x10^6$/mL
- Ideally keep spinner flasks at half volume marked on sides, can go up too (necessary for larger flasks)
- After ~P40, thaw new cells
-

To set up large spinners for aeration

- Take rounded blue aeration set up (blue, about smart phone sized) connect its thin tubing to black regulator
- Connect larger diameter tub to metal rods within larger flasks, add yellow rimmed filters to other end
- Connect filter to thin aeration tubing

  Large flask metal rod + thicker tubing + filter + small section thin tubing + black regulator + thin tubing from blue aeration tool.
- Extra tubing, filters, and regulators should all be in insect room. Additional filters near tissue culture microscope, they are not reusable.

To clean spinners

- Run distilled water through flask for 5 minutes (or rinse three times, not preferred)
- Add 10% bleach to flask, disassemble impellers in larger flasks. Let sit 1 hour or O/N.

  only leave metal tube in larger flasks in bleach for 1 hour
- If scum is present on sides of flasks, use scrub brush to remove
- Run distilled water through flask for 5 minutes (or rinse three times)
- Use aluminum foil to cover openings, loosen caps, and autoclave once (30min sterilze + 25 dry)
- Close lids and let cool in insect room on shelves

Notes

- Store all media at 4ºC and protect from light
- If working with secreted proteins, add 0.5 – 2% FBS to media during Sf9 growths
- Innoculum required, in mL = [MOI x (number of cells)]/titer – we don't do the plaque assay

  Titer ~=$1x10^6$ – $1x10^7$ – $1x10^8$

  MOI: use 0.05 – 0.1 for virus; 1 – 5 for protein expression

- Weiqiang Li had luck using just ISFM through whole process (small scale transfection etc), need to confirm
- Yana Li has insect cells kept in commercial media, passage 4x through ours to adapt
- Spinners under 125mL volume do not aerate well, don't use. Use multiple flasks instead.
- Spinners don't stay sterile for a long time, if it's been a while autoclave before use.
- Sf9 cultures won't exceed $2x10^6$/mL in smaller spinners, can reach 4 - $5x10^6$/mL in large spinners

2.2 General Nanodisc Packaging Protocol

Developed by Lily Raines, modified from Sligar and Springer Groups for packaging active tEGFR

Notes

1. Expect half of protein to precipitate during packaging step – plan accordingly for downstream applications
2. Protocol produces ~10% packaged discs to reduce formation of multiply packed discs, do not increase relative proteins amounts.

General Packaging

1. Form lipid film on interior of disposable glass culture tube by drying lipid stock solution in chloroform under gas (air/N2), then under high vacuum O/N to remove residual chloroform.
2. Resuspend lipid in cholate/TrisHCl/NaCl/EDTA solution, form lipid-detergent micelles
3. Add membrane scaffold protein (MSP) to desired MSP:lipid ratio. Add protein to incorporate if desired.
4. Incubate lipid/cholate/protein for 15 – 60 minutes at 4ºC.
5. Remove detergent with adsorbent beads (BioRad BioBeads) overnight at 4ºC.
   Note: For 100uL reactions, use a minimum 25mg biobeads [500mg/2mL reaction]
6. Remove beads from solution, filter solution with 0.45µm filter,
7. If desired, separate packaged and empty discs using on gel filtration

Lipid Film Formation

Dry 30uL of 25mM stock of lipids in chloroform (0.75 µmoles lipid)
   Note: only need about 5uL per reaction, but that's too small a volume for me to measure accurately
Resuspend with 7.5uL 200mM cholate (1.5 µmoles cholate) and 262.5µL MSP buffer
   Note: final volume 270uL, enough for ~ 6 tEGFR reactions

EGFR/Nano packaging

$7.5x10^{-4}$ µmoles scaffold protein (MSP)
43.4uL lipid resuspension
10ug EGFR ($7.\ 5x10^{-5}$ µmoles)
MSP buffer to 100uL reaction volume
[EGFR] = 0.1mg/mL

References

Ritchie TK, et al. Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. Methods Enzymol. 2009;464:211-31.
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2.3 Heterodimer Purification Protocol

Developed by Lily Raines
Approximate Time to Completion: 2 weeks
Approximate Yield: 100 – 200ug/2L cells (ErbB2/3)
All purification steps, aside from filtering, performed at 4^{o}C

1. Transiently transfect mammalian cells in suspension, using 200ug of maxiprepped DNA/L of cells with 600ug of PEI.
2. Next day, split cells back 1:3 in hybridoma media + 0.5% FBS
3. Harvest media with 1000g x 5min spin at 4oC
4. Sterile filter media w/ 0.22um filters,
    Note: Add 0.5M sodium azide to 1mM if purification does not begin immediately. Stocks above 0.5M sodium azide cannot be disposed of down the sink – do not make.
5. Use the centramate to concentrate media and buffer exchange into buffer A
    Buffer A: 20mM Tris pH 8, 1M NaCl
6. Equilibrate 5mL of nickel resin with buffer A, allow to batch bind concentrated media overnight with stirring.

- - -

7. Apply resin and media to a gravity column, collect flow-through
8. Wash resin with 10 column volumes (CV)/50mL of
    i. Buffer A + 1M Urea
    ii. Buffer A + 0.75% TX-100
    iii. Buffer A + 1M Urea
    iv. Buffer A + 0.75% TX-100
    v. 100mL of buffer A
8. Elute resin with 60mL buffer A + 250mM imidazole
    Optional: confirm elution on SDS-PAGE – do first run, not necessary thereafter
9. Dilute eluate to 1L with buffer A,
10. Equilibrate 5mL of nickel resin with buffer A, allow to batch bind diluted eluate overnight with stirring.

- - -

11. Apply resin and media to a gravity column, collect flow-through
12. Wash with 300mL of buffer A
13. Elute with 60mL buffer A + 250mM imidazole, collecting 5mL fractions
14. Run SDS-PAGE gel to determine which fractions contain heterodimer

- - -

15. Pool fractions containing heterodimer
    To detag: add excess PreScission (1-2mL Leahy Lab stock)
    To deglycosylate: add 1mL PNGaseF (Leahy Lab stock)
16. Dialyze sample for two nights against 4L of 20mM Tris **7**, 150mM NaCl, 1mM EDTA
17. To prepare for "reverse nickel," dialyze sample against 4L of 20mM Tris 8, 150mM NaCl for at least one hour
18. Equilibrate Ni-HiTrap 5mL column on Akta with 20mM Tris 8, 150mM NaCl

19. Spin sample at maximum speed for 15 minutes before loading onto Akta
    Note: you will probably need to transfer your solution to a fresh tube and spin at least one more time to remove all precipitate.
20. Collect flow-through
21. Wash with 5 CV (25mL) of 10% elution buffer - buffer A + 250mM imidazole
22. Elute column with 10 CV of buffer A + 250mM imidazole, collecting 5mL fractions
23. Run a gel and pool heterodimer containing fractions and flow-through
    WILL have some in elution fractions
24. Dialyze overnight against 4L of 20mM Tris 8, 50mM NaCl

- - -

25. Clean and equilibrate 5mL HiTrap Q column with 20mM Tris 8, 50mM NaCl
26. Spin dialyzed sample and apply to Q column
27. Collect 5mL fractions and elute using buffer A
28. Run a gel to determine which fractions contain heterodimer

- - -

29. Concentrate heterodimer containing fractions to $\leq$15mL
30. Equilibrate S200 26/60 gel filtration column in 5mM Tris 7.5, 150mM NaCl
    Note: This takes 3-4 hours and can be done overnight
30. Spin and load sample onto S200 column
31. Run a gel to determine which fractions contain heterodimer
32. Concentrate as appropriate for downstream applications.

2.4 Centramate Care and Use Protocol

Important Notes

1. NEVER fully close the retentate valve
2. NEVER touch the middle of the filter
3. NEVER start a run without finishing the run in the same 4 hour period.
4. NEVER run the centramate unattended after introducing your sample
5. The tubing used on this system is prone to breaking within the peristaltic pump; each ~6"section may only be used for 2 sample runs. Use fresh or 1x used tubing for your sample run, and mark your used section of tubing after use.
6. DO NOT leave the apparatus fully assembled unless you are 100% CERTAIN it will be used in the next two days.
7. Your sample and all buffers MUST be filtered through a 0.2 um filter.
8. Molecular weight cut-off for the filter is 30kDa, make sure the MW of your protein is above this.
9. YOU MUST PERFORM AN NWP TEST BEFORE YOUR RUN

Materials Necessary

2L PBS
16L water (re-filling two 4L buckets as necessary works well)
1L (at least) of the buffer you'd like to exchange into.
1L 0.5M NaOH
1L 0.1M NaOH
0.2um filtered sample
A timer

NWP Test Protocol

NWP: Normalized Water Permeability
TMP: Transmembrane Pressure

[(Pressure Left Front and Back)/2] - (Pressure Right Waste Valve)

1. Pump system into air, setting 200 (~1.2L/min flow)
2. Introduce distilled water to system, both valves open, run until no more bubbles seen in lines
3. Purge 3 times

   To purge: while running water, tighten retentate valve (left back) briefly until pressure rises to ~20psi. Immediately release, repeat 3x or until no more air bubbles are seen.
4. Record outlet flow over 30 seconds noting pressure on left valves
5. Pause system, tighten retentate (left back) valve slightly until pressure increases, never going above 60psi total pressure for front and back (inlet and retentate) valves.
6. Adjust TMP and collect a total of three flow rates at TMP ranging between 15 and 30psi
7. Record TMP and Flow Rates in provided table. Plot approximate data.

8. System is ready for use if NWP test results over three TMP values are between first use (maximum for filter) and first drop (dramatic drop seen after initial filter run) lines.

Concentration and Diafiltration Protocol

1. Assemble Apparatus

Note: Only touch the sides of the filter and NEVER the middle

Note: If you match the orientation of holes in the gasket, you will place the filter in the correct orientation.

a. Remove gasket from 0.1M NaOH storage solution, place onto centramate system.

b. Add filter, then sandwich with remaining gasket.

c. Top with metal plate.

d. On each of the 4 protruding screws, add a longer metal rod, a shorter metal rod, a washer, and a hex nut.

e. Use a torque wrench to fasten the metal plate. Confirm that this wrench is set to 70psi and is in the 'on' position. DO NOT tighten one screw completely and then complete the others. Tighten one screw slightly, then the screw directly across from it, etc, until torque wrench 'breaks' while tightening. This will prevent you from over-tightening the system.

f. Feed inlet tubing through peristaltic pump. Make sure tubing is placed in tightly. Confirm that pump speed is set to 200. This corresponds to ~1.2L/min flow out of the pump, which will translate to 0.5L/min off the filter.

2. Pump System into air

3. Rinse System into water

a. Run water through system until no bubbles are seen

b. Purge valves (same as with NWP test)

c. Run 8L of water through the system, do not recirculate (do not place retentate and permeate tubing in feed vessel).

4. Perform NWP Test (see earlier protocol)

5. Rinse System into 1L PBS

a. Run PBS through system for 30 seconds, without collecting retentate or permeate

b. Recirculate (place inlet, retentate, and permeate tubing in same feed vessel) remaining PBS for 10 minutes.

5. Concentrate Media

After starting this step, DO NOT LEAVE DEVICE UNATTENDED until sample is harvested

a. Place inlet and retentate tube in feed vessel with media.

b. Start pump, proceed 'as low as you can go', until no more media can be taken up without introducing air into the system

NOTE: you MAY pause the pump briefly during this process (steps 5-7), but only to add media/buffer or to switch to a smaller feed vessel.

6. Diafiltrate
    a. Pause pump, add new buffer to concentrated sample.
    b. Restart pump with inlet and retentate tubing in same feed vessel.
    c. Diafiltration with 1L (10 x dead volume) will result in >96.8% buffer exchange.
7. Harvest
    a. Close permeate valve, keep retentate valve fully open
    b. Pump system into air and collect retentate

Note: if your protein is especially sensitive you can harvest with PBS, but this will limit your final volume.

8. Rinse system into 1L PBS and recirculate
    a. As in step (3), run PBS through system until no bubbles are observed, purge valves (unless already did this with additional buffer chase)
    b. Recirculate (inlet, retentate, and permeate tubing in same feed vessel) for 10 minutes.
9. Rinse system with 8L of water without recirculating
10. Recirculate 1L 0.5M NaOH in system for 30 minutes
    a. Let 0.5M NaOH run through system for ~10sec before starting recirculation to flush out residual water

11. Storage
    If less than two days before next use:
        Introduce 0.1M NaOH to the system. Ensure no bubbles are present, store retentate, permeate, and inlet tubing in feed vessel.
    If more than two days:
        Run system into 0.1M NaOH, then into air. Disassemble filter, turning torque wrench to the ‘off’ position, store in provided airtight container of 0.1M NaOH (if debris noticed in storage solution, replace 0.1M NaOH). Loosen all gauges and joints. Flip torque wrench back to ‘on’ position and ensure it is set to 70psi.

2.5 Monthly AKTA Maintenance Protocol

Developed by Jennifer Kavran, typed by Lily Raines

Once a month, perform the following maintenance on all AKTA's

1. Wash both pumps into 1M NaOH
2. Run NaOH through system, set to 50% B to flow through both pumps, for 30 minutes at 1 mL/min
3. Microwave approximately 1L of MilliQ water for 15 minutes
4. Pump wash into hot MilliQ, run water through both pumps for at least 30 minutes at 5mL/min
5. Check pH of waste – if neutral, proceed
6. Change 20% ethanol at pump seals
    - at AKTA Pure, in a 50mL conical tub at bottom right of instrument. Change tube itself if grimy
    - at other AKTA, ethanol is in a square plastic bottle. **Be gentle with four inlet tubes** (two per pump) while re-filling, cover with parafilm

* tubing becomes fragile and can break over time. If tubing does break, order replacement from GE. Expect 2 – 3 days for it to arrive

7. Remove unlabeled buffers and tubes from deli cases/AKTA cabinets

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Curriculum Vitae

Full Name
Lily Lambert Raines

Place of Birth
Baltimore, Maryland. September 10, 1988

Educational History

Ph.D. expected	2016	Program in Biochemistry, Cellular, and Molecular Biology Mentor: Daniel Leahy, Ph.D.	Johns Hopkins, School of Medicine
B.S., high honors	2010	Biochemistry, Spanish Minor	Eckerd College

Research Experience

Ph.D. Candidate 2010 – 2016 Johns Hopkins, School of Medicine
Mentor: Daniel Leahy, Ph.D.
Topic: Using Biochemical and Structural Approaches to Study EGFR-family Heterodimers

Research Rotation	2010	Lab of Caren Freel Meyers, Ph.D.
Research Rotation	2010	Lab of Svetlana Lutsenko, Ph.D.
Internship	2009	Walter Reed Army Institute of Research
Internship	2008	Goddard Space Flight Center
REU Fellowship	2007	Auburn University

Teaching Experience

Course Director	2014	Effective Science Communication, School of Medicine
Curriculum Writer and Teacher	2014	Science Outreach Program
Teaching Assistant	2014	Scientific Foundations of Medicine: Macromolecules Section, School of Medicine
High School Student Mentor	2011 – 2014	Incentive Mentoring Program/Thread
Rotation Student Mentor	2013	Lab of Daniel Leahy, Ph.D.
Summer Teaching Camp	2013	Institute for Excellence in Education, School of Medicine

Other Professional Experience

Science Outreach Internship	2015	The American Society for Biochemistry and Molecular Biology

Service and Leadership

Lead Science Café Organizer	2013 – 2015	Project Bridge
Workshop Volunteer	2013 – 2015	Project Bridge
Colloquium Organizer	2013 – 2014	BCMB Program

Scholarships, Fellowships, or Other External Funding

NIH NCI	NRSA 1F31CA180395	2013 – 2016	$126,696
Biomedical Careers Initiative	Travel Award	2015	$500

Academic and Other Honors

2010	Phi Beta Kappa	Phi Beta Kappa
2009	Barry M. Goldwater Scholarship	Barry Goldwater Scholarship and Excellence in Education Foundation
2006-2010	Dean's List	Eckerd College
2009	Undergraduate Award in Analytical Chemistry	Eckerd College
2009	Spirit of Service Award	Florida Senate
2007	Chemistry Achievement Award	CRC Press

Publications

Wang Z, **Raines LL**, Hooy RM, Roberson H, Leahy DJ, Cole PA. Tyrosine phosphorylation of mig6 reduces its inhibition of the epidermal growth factor receptor. ACS Chem Biol. 2013 Nov 15;8(11):2372-6. PMCID: PMC3830645

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Poster Presentations

Raines LL, Leahy DJ (2015) Using Biochemical and Structural Approaches to Study Erbb2- Containing Heterodimers. Science Communication and Outreach Career Symposium, San Antonio, TX, September 19 2015.

Raines LL, Leahy DJ (2015) Using Biochemical and Structural Approaches to Study Erbb2- Containing Heterodimers. 45th Mid-Atlantic Protein Crystallography Meeting, Baltimore, MD, June 4 2015.

Raines LL, Leahy DJ (2015) Using Biochemical and Structural Approaches to Study Erbb2- Containing Heterodimers. Biophysical Society 59th Annual Meeting, Baltimore, MD, February 10 2015.

Raines LL, Hudson RL, Moore MH (2008) Following the Carbon: Structure, Chemistry, and Spectroscopy of Frozen Ethane. Abstract for poster presentation. 40th Annual Meeting of the Division for Planetary Sciences of the American Astronomical Society. Cornell University, Ithaca, New York. October 13, 2008.